

Studies on Human Serum Cholinesterases.

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Summary.

The molecular, enzymic and physiological characteristics, methods of assay and the clinical significance of human serum cholinesterase are reviewed. The existence of multiple forms of enzymes and the particular inherited polymorphism of human cholinesterase which results in abnormal responses to the muscle relaxant, suxamethonium, are summarised.

The effect of reaction temperature on the hydrolysis of substrates by cholinesterases has been investigated and significant differences have been demonstrated. The temperature/activity relationships have been found to reflect the sensitivity of the different phenotypes to suxamethonium. At the physiological temperature of 37°C the cholinesterases of the three phenotypes which exhibit an abnormal response to the drug have been shown to undergo a pronounced reversible inactivation. This suggests that the prolonged response to suxamethonium in these cases is not entirely due to the reduced enzymic affinity but is in some part caused by the inactivation of the enzyme species at its functional temperature. This in turn indicates that only by conducting assays at 37°C is a valid indication of serum cholinesterase activity obtained and also explains the anomalies recorded in the literature concerning activities obtained at different temperatures.

Thermodynamic data derived from these studies indicate that both genes regulate enzyme synthesis at equal rates, that is, heterozygous sera contain equal molecular proportions of both variants. The same data is employed to

show that the different molecular activities of the isoenzymes on this equal synthesis basis explains the skewed distributions of inhibitions in heterozygote sera.

The methods of differentiating the allelic isoenzymes of human serum cholinesterase by inhibitions have been examined and extended. The use of succinylcholine as a differential inhibitor reveals anomalies in the limited number of allelic variants which are currently recognised. It is postulated that a larger number exists. The action of some non-depolarising muscle relaxant drugs on enzymic hydrolysis has also been investigated in vitro. Of these, pancuronium has been shown to inhibit not only human serum cholinesterase but also erythrocyte acetylcholinesterase.

The effect of reaction temperature on the inhibition of the cholinesterase isoenzymes by a variety of agents has also been examined. Variation with type of inhibitor and allelic variant has been demonstrated and because of this the ability to precisely categorise a phenotype is only possible at the definition temperature, that is, about 25°C. The anomalous inhibitions found at this temperature for the "fluoride-resistant" enzyme are explained by temperature sensitivity and at 37°C the inhibitions accord with the observed drug response of this variant.

By means of inhibitions, by relative substrate hydrolysis rates or by ratios of activities at two reaction temperatures, techniques have been evolved, employing thiocholine ester substrates capable of screening large numbers for "suxamethonium sensitivity".

Population surveys gave no direct support to the

postulate of numerous cholinesterase variants. It has been found however that there is an apparent modification of the "fluoride-resistant" isoenzyme during pregnancy. This indicates how readily a new identifiable variant can be produced and opens avenues for further elucidation of the changes in molecular structure which result in recognisable polymorphism.

1. Introduction.

(a) Cholinesterase.

(a) Cholinesterase (E.C.3.1.1.8 Acylcholine acyl-hydrolase).

The term "choline-esterase" was first used by Stedman, Stedman and Easson (1932) to describe an enzyme which hydrolysed acetylcholine (Engelhart and Loewi, 1930), the substance which is liberated at the motor end plates during a nerve impulse. The importance of the enzyme in the physiology of the nervous system was readily realised for by hydrolysing the acetylcholine after transmission it permitted the nerve fibre to accommodate the next impulse. What was not appreciated until the studies of Alles and Hawes (1940) was that there are two enzymes which hydrolyse choline esters present in human tissues.

The enzyme predominating in nerve tissue and present in erythrocytes has variously been termed "specific", "true", "aceto-" or "e-type" cholinesterase in distinction to the "non-specific", "pseudo-", "butyro-" or "s-type" cholinesterase found in blood serum and synthesised by the liver. These hydrolases have wide and overlapping substrate specificities but the former has a specificity directed towards acetyl esters and is correctly termed acetylcholinesterase (E.C.3.1.1.7 acetylcholine acetyl-hydrolase) while the latter, cholinesterase, is active against choline esters and a few other compounds and is given the systematic name acylcholine acyl-hydrolase. (E.C.3.1.1.8).

Since there are innumerable naturally occurring esters there must be enzymes capable of hydrolysing all of them. An elementary knowledge of protein structure denies the possibility of one enzyme being capable of hydrolysing all,

and equally it can be inferred that there is not an enzyme for each ester since a protein capable of binding and activating one will also be effective against other esters with closely related electronic configurations. Thus most of the simple choline esters are also hydrolysed by the so-called ali-esterases which act on aliphatic esters and glycerides. Similarly compounds such as benzoylcholine or phenylacetate which are substrates for cholinesterase are also hydrolysed by the aryleresterases which are present in human blood plasma. However both acetylcholinesterase and cholinesterase are extremely sensitive to eserine (physostigmine) being completely inhibited at a concentration of 10^{-5} M and it is upon this rather tenuous point that the specific existence of these enzymes is recognised.

Acetylcholinesterase and cholinesterase are readily differentiated from each other by specific substrates such as acetyl- β -methyl choline for acetylcholinesterase and benzoylcholine for cholinesterase. Differentiation can be achieved even by substrate concentration; acetylcholinesterase is inhibited at concentrations of acetylcholine greater than 10^{-3} M at which levels cholinesterase exhibits maximal activity. Selective inhibitors have also been employed, tri-*o*-cresyl phosphate for cholinesterase (Mendel and Myers, 1952) and $\beta\beta'$ -dichlorodiethyl-N-methylamine for acetylcholinesterase (Adams and Thompson, 1948). In any case since the present study was mainly concerned with investigation of human blood the two enzymes are already separated, acetylcholinesterase in the erythrocytes and cholinesterase in the plasma or serum.

Molecular Properties

Wilkinson (1970) gives the molecular weight of cholinesterases as $2-12 \times 10^6$ without however quoting the source of this information. Certainly the molecular weight of human serum cholinesterase is greater than 200,000 since it appears immediately after the void volume in gel filtration on Sephadex G-200. After purifying human serum cholinesterase 3400-fold Surgenor and Ellis (1954) found the preparation to contain four protein fractions with the major part of the activity in the 10 S fraction with a molecular weight calculated as 300,000. With material purified 10^4 times from human serum Haupt, Heide, Zwisler and Schwick (1966) found a molecular weight of 348,000 and measured immunologically the serum concentration of cholinesterase was about 9 mg/litre. With an even more highly purified preparation Das and Liddell (1970) calculated a molecular weight of 366,000 from ultracentrifuge data. Jansz and Cohen (1962) by titration with diisopropylfluorophosphate (DFP) calculated a molecular weight of 84,000 per active site. From these data it would appear that the cholinesterase molecule possesses four active sites.

From ethanol-water solubility studies Surgenor, Strong, Taylor, Gordon and Gibson (1949) estimated the isoelectric point of human serum cholinesterase to be pH 4.6-4.9. Svensmark (1961) showed the enzyme to be an acid glycoprotein containing several sialic acid residues and in a classical paper electrophoresis study, Svensmark and Kristensen (1963) showed the isoelectric point of native human cholinesterase to be pH 2.9-3.0, that of neuraminidase treated enzyme

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pH 6.7-7.0. These experiments showed that at pH 8.6 native human cholinesterase behaves as an α_2 globulin migrating slightly more slowly than the main fraction. At pH 11.0 the enzyme behaves as a β -globulin at pH 6.0 as an α_1 globulin and at pH 5.0 as an albumin. At a pH lower than 4.7, the isoelectric point of albumin, cholinesterase can therefore be expected to migrate as a pre-albumin fraction. Das and Liddell (1970) found an isoelectric point of pH 3.99 on a high purified enzyme sample by isoelectrofocusing technique.

Electrophoresis of human serum on starch gel has resulted in contradictory reports. Thompson and Cook (1961) and Ecobichon and Kalow (1962) found one fraction while one or more weaker bands have been recorded by Dubbs, Vivonia and Hilburn (1960) Bersohn, Barron and Hess (1961), Harris, Hopkinson, Robson and Whittaker (1963). Harris and co-workers employed two-dimensional electrophoresis at pH 8.6 on filter paper for the first run followed by starch gel in the second. In fresh normal serum four zones of activity termed C_1 to C_4 were separated migrating on paper between the α_2 and β globulins, C_2 slightly fastest. The C_4 fraction which was slowest moving in both directions contained about 90% of the total activity. All four fractions were sialo-proteins and mobilities were reduced by neuraminidase treatment (Harris, Hopkinson and Robson, 1962). Fresh foetal or cord blood had an additional zone overlapping the C_4 fraction and after storage two more minor, faint and fast-moving spots appeared.

La Motta, McComb and Wetstone (1965) also separated

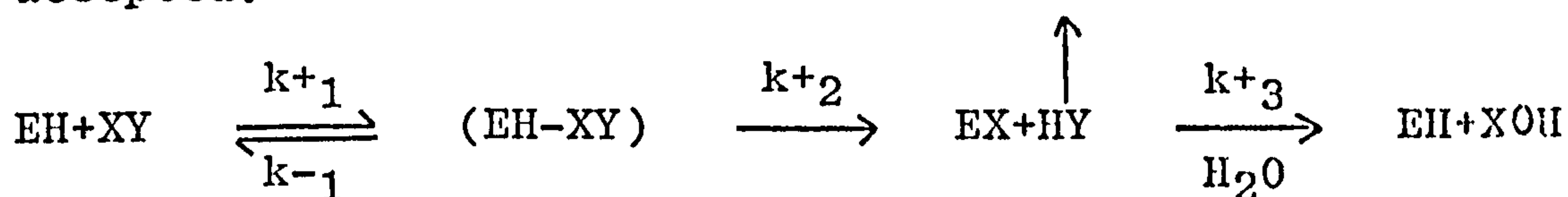
five cholinesterase fractions from human serum concentrates by starch-gel electrophoresis. The forms were found to be interconvertible and multiplicity was attributed to polymerisation. Two further fractions were later reported by La Motta, McComb, Noll, Wetstone and Reinfrank (1968) which like the others could be converted into a single fraction by concentration. Harris and Robson (1963) reported that the C_1 to C_4 fractions isolated by starch-gel electrophoresis could also be separated in reverse order by gel-filtration through a Sephadex column thereby confirming that the molecular sizes increased from C_1 to C_4 . Despite these findings a simple polymerisation is doubtful as a single explanation of the heterogeneity since it does not account for the increased mobility of the C_2 fraction on paper electrophoresis. This fraction and the foetal cholinesterase may differ in sialic acid content.

Enzyme Characteristics

The sialic acid moieties are in no way concerned with the catalytic properties of serum cholinesterase. Removal of the sialic acid does not alter the enzyme activity and it has been shown (Svensmark, 1961; Augustinsson and Eskedahl, 1962) that carbohydrate-free serum cholinesterase is identical to the native enzyme with regard to neostigmine inhibition and to hydrolysis of acetylcholine, propionylcholine and butyrylcholine as determined by Michaelis constants. Dibucaine and fluoride inhibitions of native and neuraminidase-treated cholinesterase is identical (Echibicon and Kalow, 1963).

No prosthetic groups have been proposed or found for serum cholinesterase and there is no evidence that the enzyme is a metalloprotein. Ammon (1943) however suggested that magnesium or calcium ions were required for maximum activation and some reduction in activity was found on removal of these ions and by addition of fluoride, oxalate or citrate.

The three-stage mechanism of hydrolysis by cholinesterase elaborated by Davies and Green (1958) in which the last two stages are not reversible is now generally accepted.



Where EH is the enzyme, XY the ester, EH-XY the Michaelis-Menten complex and EX the acylated enzyme.

To explain the affinity of choline ester hydrolases for cationic substrates and inhibitors (Figure 1), Zeller and Bissegger (1943) postulated the presence of an "anionic" and an "esteratic" site at the active centre of acetylcholinesterase. Since cholinesterase was not inhibited by high substrate concentrations it was assumed to have only an esteratic site. Wilson (1954) suggested the presence of both sites in the active centre of cholinesterase and Bergmann (1958) explained the substrate inhibition by attributing two anionic sites to acetylcholinesterase. The problem is still controversial.

The amino-acid sequence of a peptide assumed to come from the active centre by its binding to labelled diisopropylfluorophosphate has been analysed (Cohen, Costerbaan,

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Jansz and Berends, 1959) and found to be similar to that of a large group of hydrolases containing a serine residue with one of the dicarboxylic aspartic or glutamic acids on one side and glycine or alanine on the other (Sanger, 1963).

Assay Procedures

For the assay of cholinesterase activity, procedures of every type exist, fixed-time, fixed end point and initial reaction rate methods. In general these assays may be divided into three categories. First there are those measuring the liberation of acid, by estimating the carbon dioxide released from a bicarbonate-containing medium in the Warburg manometric apparatus (Ammon, 1933; McArdle, 1940), by the time taken to achieve a fixed pH electrometrically (Michel, 1949; Courville and Ledington, 1951), or colorimetrically by means of such indicators as phenol red (Reinhold, Tourigny and Yonan, 1953; Molander, Friedman and LaDue, 1954; Caraway, 1956), bromthymol blue (Biggs, Carey and Morrison, 1958) or m-nitrophenol (Rappaport, Fishl and Pinto, 1959), or by rate of manual (Stedman, Stedman and Easson, 1932; Glick, 1938; Alles and Hawes, 1940) or automatic (Jorgensen, 1959; Nabb and Whitefield, 1967) continuous titration to maintain a constant pH.

In the second group use is made of chromogenic substrates such as β -carbonaphthoxycholine, coupling the liberated β -naphthol with tetrazotised diorthoanisidine (Ravin, Tsou and Seligman, 1951) or non-choline esters such as phenylbenzoate with which the phenol liberated by enzymic hydrolysis is estimated with the Folin-

Ciocalteau reagent (Smith, Lowenthal, Lehmann and Ryan, 1959). Reaction rate procedures with substrates of doubtful specificity for cholinesterase such as indophenylacetate at 625nm (Kramer and Ganeson, 1958) or o-nitrophenylbutyrate at 414nm (Main, Miles and Braid, 1961; McComb, La Motta and Wetstone, 1965; Szasz, 1968; Whittaker and Hardesty, 1969) have also been published. Perhaps the most generally successful approach has been that using thiocholine esters and the estimation of the sulphhydryl released by enzyme hydrolysis. This has been followed by means of the nitroprusside colour reaction (McOsker and David, 1959) or by iodometric titration (Augustinsson, 1955) in two-point assays or in reaction rate procedures by coupling to the reduction of 2:6 dichlorophenolindophenol reading at 600nm (Gal and Roth, 1957); by complexing with 2'2' or 4'4' dithiodipyridine and reading at 324 or 343nm respectively (Uete et al., 1972) or with 5,5'-dithiobis (2-nitrobenzoic acid) following the reaction at 410nm (Ellman, Courtney, Andres and Featherstone, 1961; Garry and Routh, 1965; Szasz, 1968a, b; Garry, 1971). The last is a most simple and sensitive procedure.

In the final group the unreacted ester is estimated. This has been done in fixed-time assays employing an acetylcholine substrate by means of the ferric-hydroxamate reaction (De La Hueraga, Yesinick and Popper, 1952) or in reaction rate procedures with thiocholine substrates using absorption of the ester at 226nm or with benzoylcholine by following the change in absorption at 240nm (Kalow and Lindsay, 1955). The last method is of particular

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interest because of the recognised specificity of the substrate, the fact that it is an initial reaction rate assay and was found to have an excellent precision. Further reasons for interest in this assay are expanded upon in the next section.

Methods for the determination of cholinesterase activity have recently been reviewed by Augustinsson (1971).

Physiological Function

Plasma cholinesterase is synthesised in the liver but cholinesterases are widespread in human tissues, in intestine, muscle, lung, uterus, pancreas and other glands and in certain ganglia. Cholinesterase is present in lymph and effusion fluids. Activity is detectable in cerebrospinal fluid but not normally in urine, bile, gastric juice or saliva.

The physiological role of cholinesterase and its natural substrates are matters for speculation. At its simplest Funnell and Oliver (1965) have suggested that cholinesterase plays a part in the homeostatic mechanism controlling the levels of free choline. Again since butyrylcholine, which has a possibly undesirable nicotinic effect, may be synthesised by the choline acetylase system from butyryl- coenzyme A during fatty acid metabolism, Clitherow, Mitchard and Harper (1963) have speculated that the function of the enzyme may be to prevent accumulation of this substance. Similarly γ -aminobutyryl choline which is present in brain tissue may be a substrate for cholinesterase (Holmstedt and

Sjoquist, 1960).

Perhaps the discovery that acetylcholinesterase and cholinesterase were different enzymes, the former in high concentration at neural junctions was misleading since it lead to the assumption that cholinesterase was not concerned with nervous transmission (Mendel and Rudney, 1943, 1944). Certainly the lack of pharmacological reaction to selective cholinesterase inhibition (Hawkins and Gunter, 1946; Mazur and Bodansky, 1946) appeared to confirm this but more recent work has shown that such inhibition affects intestinal motility (Jamieson, 1963) and the enzyme may play a part in transmittory processes in other tissues also (Koelle, 1963).

However speculation concerning the role of cholinesterase must take into account that normal healthy individuals have been found devoid of serum cholinesterase activity from which it would appear that the function of the enzyme is not critical or else can be taken over by alternative mechanisms.

Following enzyme blocking with diisopropylfluorophosphate Neitlich (1966) has calculated from the generation curve that the biological half-life of cholinesterase is ten days. This value has been corroborated independently by Jenkins, Balinsky and Patient (1967) from the rate of disappearance of transfused normal plasma activity into a patient lacking plasma cholinesterase.

Clinical Significance

The activity of most enzymes in human blood plasma is low and their presence there in health is a result of

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normal tissue wear and tear. They are cellular detritus and form part of the circulating protein pool. In clinical enzymology it is an increase in the activity of these enzymes which is sought, an increase which would indicate an abnormal efflux into the circulation as a result of some pathological situation. Apart from the enzymes of the coagulation mechanism, cholinesterase is the only enzyme of interest in clinical enzymology which may be termed plasma or serum specific. As such cholinesterase is normally present in human blood serum in relatively high concentration and it is a decrease in activity which is clinically meaningful. Further, since cholinesterase is synthesised in the liver, it should be a useful index of the protein synthesising function of the liver. In this respect it is very similar to serum albumin and indeed there would appear to be a concomitance in the synthesis of these two proteins.

Hence in hepatitis, liver metastases (Molander, Friedman and La Due, 1954; Burnet, 1960) and cirrhosis (Kunkel and Ward, 1947; La Motta, Williams and Wetstone, 1957; Hunt and Lehmann, 1960) where there is a lesion of the synthesising mechanism, low levels of both cholinesterase and albumin are found in serum. In obstructive jaundice both are essentially normal (Antopol, Schifrin and Tuchman, 1938; McArdle, 1940; Burnett, 1960) except where the causative factor is malignant (Mann, Mandel, Eichman, Knowlton and Sborov, 1952; Williams, La Motta and Wetstone, 1957). The last instance is probably a reflection of the effect of the tumour on protein metabolism and, like

malnutrition (Vorhaus and Kark, 1953), the low serum levels are a result of depletion of material for synthesis. The only lack of coincidence occurs in nephrosis, in which albumin, the much smaller molecule, but not cholinesterase is lost through the damaged kidney. The compensatory increase in protein synthesis results in high serum cholinesterase activity in the presence of low serum albumin levels (Kunkel and Ward, 1947; Vorhaus and Kark, 1953; Vincent, 1958). The finding of normal albumin levels in individuals having a total lack of serum cholinesterase activity or of normal cholinesterase activity in analbuminaemia (Dietz, Rubinstein and Lubrano, 1966) in no way negates the speculation that there is an intimate connection between the hepatic synthesis of albumin and serum cholinesterase.

In myocardial infarction there is a characteristic fall in serum cholinesterase activity (Oka, 1954) which reaches a minimum about the fifth day and then increases to normal levels; a failure to rise carries a grave prognosis (Moore, Birchall, Horack and Batson, 1957). Not only in myocardial infarction, but also in pulmonary embolism, acute infectious, surgery, indeed any precipitation of the "acute phase reaction" (Hauss and Leppelmann, 1958) gives rise to lowered serum cholinesterase activity.

Increased serum activity is found in cases of thyrotoxicosis and reduced levels in myxoedema (Thompson, 1959; Thompson and Whittaker, 1965) but the high values reported in diabetes mellitus are a reflection of obesity, not of the disease (Thompson and Trounce, 1956). In myasthenia gravis, a disease characterised by muscle weakness which

responds to eserine and prostigmine treatment, Stedman and Russell (1937) found low serum cholinesterase activities but later studies did not confirm this (Vorhaus and Kark, 1953). Stedman and Russell also noted low serum activities in progressive muscular dystrophy and myotonia congenita and Moore, Birchall, Horack and Batson (1957) reported similar results in dermatomyositis but normal levels in other collagen diseases.

Some observers have reported lowered serum cholinesterase activity in schizophrenia and increased levels in anxiety states. Plum (1960) however noted increased cholinesterase activity in manic depressive psychosis and schizophrenia as well as in anxiety and depressive neurosis but while Antebi and King (1962) observed increased levels in 20% of chronic schizophrenics, they found no correlation between the enzyme abnormality and the disease type or clinical state of the patients.

Although assay of serum cholinesterase activity would appear capable of yielding information of clinical value in the correct setting, only in Germany has it gained such recognition. This is probably a result of the fact that normal serum cholinesterase activity is so high and the range of activities so wide that unless the patient's usual activity is at the lower end of the range such variations are not readily obvious.

As it is, it is not in the diagnosis or monitoring of any pathology that assay of serum cholinesterase is most often sought but rather in the field of pharmacogenetics, that is, the study of genetic variants in man

which are responsible for alterations in the response to drugs.

Polymorphism

Some of the pharmacological properties of succinyl-dicholine (suxamethonium, Scoline (R)) were first described by Hunt and Taveaux (1906) but forty-three years were to elapse before its potential as a short-acting muscle relaxant was discovered (Bovet, Bovet-Nitti, Guarino, Longo and Marotta, 1949). Succinyl-dicholine produces a depolarising type of neuromuscular block since it acts as a competitive inhibitor of acetylcholinesterase. The short period of action, three to five minutes, results from the hydrolysis of the drug by the cholinesterase of the circulation. It was therefore to be expected that in any pathology in which serum cholinesterase was low that the duration of paralysis would be extended and indeed not long after its introduction cases were reported of prolonged apnoea following administration of usual doses of suxamethonium. However these episodes, up to two hours duration, occurred in individuals not suffering from liver disease, malnutrition or other disease which might account for their low serum cholinesterase activity (Evans, Gray, Lehmann and Silk, 1952; 1953; Bourne, Collier and Somers, 1952; Forbat, Lehmann and Silk, 1953).

A great advance in understanding of this phenomenon came from the family studies of sensitive individuals (Lehmann and Ryan, 1956; Lehmann, Patston and Ryan, 1958) which resulted in the hypothesis of an inherited enzyme defect, the enzyme level determined by two allelic genes

neither of which showed dominance. However although Calvert, Lehmann, Silk and Slack (1954) demonstrated a close correlation between suxamethonium dose and duration of paralysis, and that when the serum cholinesterase activity was doubled by injection of purified enzyme the apnoeic response was halved, patients who suffered what came to be known as "Scoline apnoea" often had serum cholinesterase levels higher than other patients who showed no unusual response to the drug. Lehmann and Simmons (1958) while confirming the hereditary nature of the phenomenon stated the anomaly as "it is often difficult to understand why patients with an identically low pseudocholinesterase level show a varying duration of apnoea following the same dose of suxamethonium; the duration may vary from no excessive prolongation to a very prolonged apnoea".

The explanation of this was unfolded by the work of Kalow and colleagues in Toronto who showed that the affinity of the enzyme in the sera of sensitive individuals for a wide range of substrates was lower than that of the serum enzyme of normal individuals (Kalow, 1959a; Davies, Marton and Kalow, 1960). Indeed the enzyme in the serum of sensitive patients had no detectable activity against succinylcholine at therapeutic concentrations. The behaviour of the two enzymes to various inhibitors also varied considerably, the normal enzyme in most cases being more strongly affected (Kalow and Davies, 1958). The compound which produced the greatest differential inhibition was dibucaine (cinchocaine, Nupercaine) at a concentration of $10^{-5}M$ and this gave a means of detecting the "atypical"

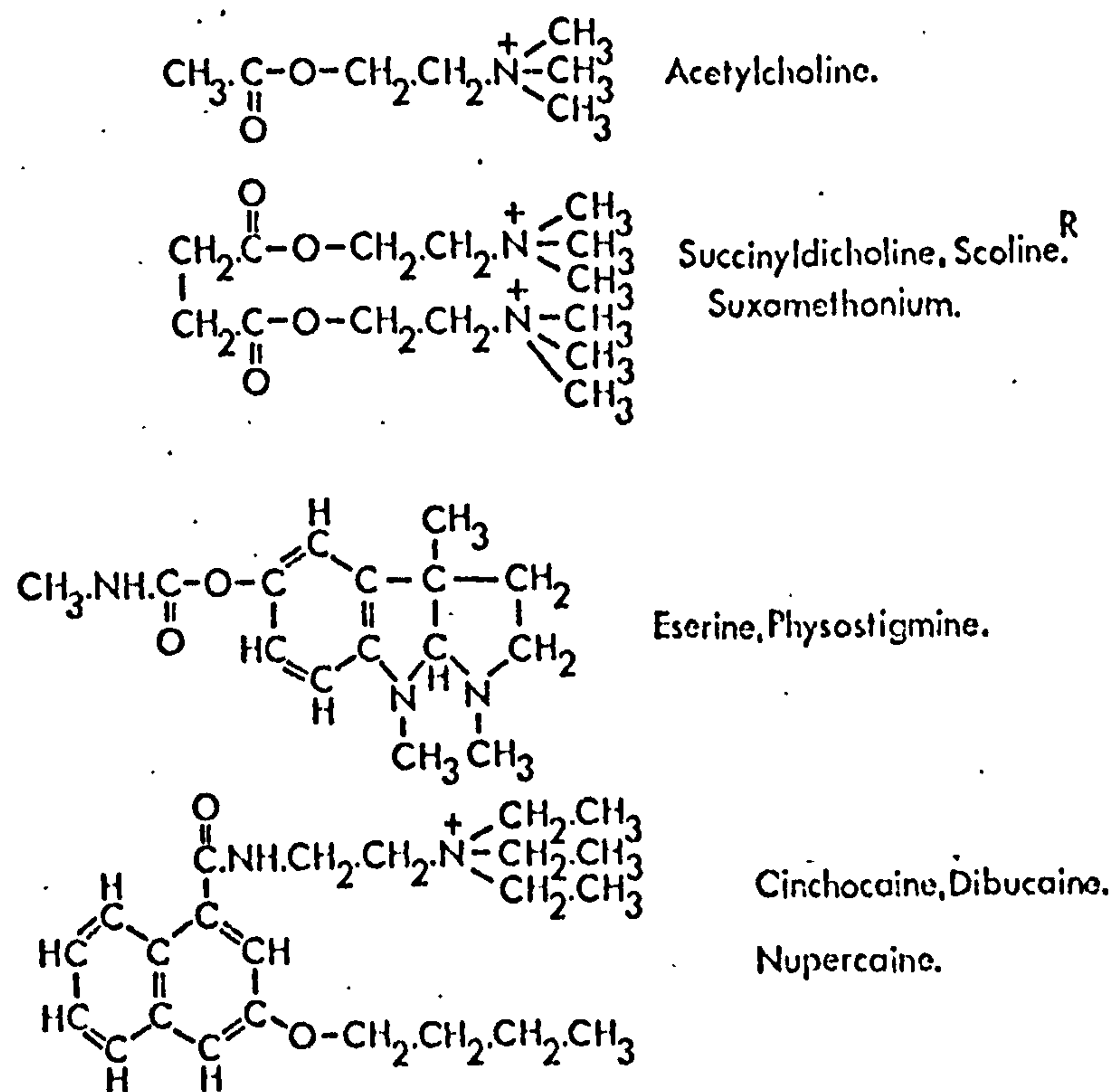


Figure 1. Structural formulae of some substrates and inhibitors of cholinesterase.

Further examples are illustrated in Figure 20.

enzyme as it was called (Kalow and Staron, 1957). With a 50 μ M benzoylcholine substrate (Kalow and Lindsay, 1955) the usual enzyme was inhibited about 80%, the atypical enzyme about 20% and heterozygous sera about 60%. The percentage inhibition was termed the dibucaine number (DN) and this procedure clearly segregated the three genotypes irrespective of the level of cholinesterase activity.

In view of the apparent particular affinity of cholinesterase for compounds containing a positively charged nitrogen atom particularly when linked to methyl groups (Figure 1) it is not surprising that most of the inhibitors examined by Kalow and co-workers contained such a positively-charged nitrogen. That this constitution was not essential in a differential inhibitor was shown by Harris and Whittaker (1961) who found that sodium fluoride provided a similar inhibition segregation to that of dibucaine. However when these workers compared the dibucaine and fluoride numbers of individuals from families of suxamethonium-sensitive patients besides the three groups already segregated, two further groups were recognisable with slightly lower dibucaine numbers and much lower fluoride numbers. Further work (Harris and Whittaker, 1962, 1963) showed that this increased resistance to fluoride inhibition is inherited through a gene allelic to the usual and atypical genes. The heterozygote for the atypical and fluoride-resistant genes is sensitive to suxamethonium (Lehmann, Liddell, Blackwell, O'Connor and Daws, 1963) as is the homozygote for the fluoride-resistant cholinesterase (Liddell, Lehmann and Davies, 1963; Griffiths, Davies and Lehmann, 1966).

Sodium chloride at high concentration (0.5M) also differentially inhibits the cholinesterase variants (Whittaker, 1968b). The chloride number differs from both dibucaine and fluoride numbers in that suxamethonium-sensitive individuals have the higher values and normals the lower figures.

Cases have also been described (Liddell, Lehmann and Silk, 1962; Hart and Mitchell, 1962; Gutsche, Scott and Wright, 1967) with little or no cholinesterase activity, attributable to a homozygous state for a "silent" gene, which is allelic with the other cholinesterase variants (Simpson and Kalow, 1964). The locus of these genes is closely linked to the transferrin locus (Robson, Sutherland and Harris, 1966).

A further serum cholinesterase variant was discovered by two-dimensional electrophoresis, the first run on paper being followed by starch-gel at pH 8.6 (Harris, Hopkinson and Robson, 1962). In normal serum four zones of activity termed C_1 to C_4 are separated. The C_4 fraction is the slowest moving and contains about 90% of the total activity. In about 10% of the British population an even slower fifth zone, C_5 , is found, which is genetically determined and possessors have on the average 25% more serum cholinesterase activity than those without it (Harris, Hopkinson and Robson, 1962; Harris, Hopkinson, Robson and Whittaker, 1963; Robson and Harris, 1966). This variant is determined by a gene acting at a locus distinct and separate from that determining the inhibitor-sensitive variants (Harris, Robson, Glen-Blott and Thornton, 1963; Simpson, 1968). The mode of

action of the C_5 gene and the relationship of the two loci are not understood.

Two conventions have been proposed for classifying the generally-recognised genes. In the first nomenclature (Motulsky, 1964) the two loci are designated E_1 and E_2 while that of Goedde and Baitsch (1964) uses Ch_1 and Ch_2 . The four variants at the first locus are symbolised E_1^u or Ch_1^U for the usual enzyme, E_1^a or Ch_1^D for the atypical (dibucaine-resistant) gene, E_1^f or Ch_1^F for the fluoride-resistant allele and E_1^s or Ch_1^S for the silent gene. At the second locus the two variants are designated as E_2^- or Ch_2^- for normals or as E_2^+ or Ch_2^+ for the C_5 variants. In that which follows, the convention of Motulsky (1964) is used exclusively.

1. (b) Multiple Forms of Enzymes.

(b) Multiple Forms of Enzymes

The knowledge that certain basic metabolic pathways such as the glycolytic system and the citric acid cycle occur, with, at most, only minor modification, in almost all living cells of animals, plants and micro-organisms would suggest that the enzymes constituting these systems are similar. This concept was supported by the knowledge that all the important prosthetic groups and coenzymes, such as pyridoxal phosphate, coenzyme A, nicotinamide-adenine dinucleotide had the same chemical structures regardless of their derivation.

The basic conviction was definitively stated by Dixon and Webb (1958).

"It is a remarkable fact that in general the catalytic properties, specificity, activity, affinities, etc. of a given enzyme vary little with the source. Although there may be slight physical differences in a given enzyme when it is produced by different cells, they are usually unimportant, and the enzyme remains essentially the same enzyme. This is in fact a commonplace of enzymology; for all practical purposes the source of an enzyme is usually regarded as a somewhat secondary matter."

This creed may still be valid for enzymologists but to the clinical biochemist the source of an enzyme is of paramount importance and any facet of enzyme existence which will disclose this origin is earnestly examined. Again, to the geneticist, any ontogenic or phylogenic variation in a specific enzyme is meaningful.

As Fischer (1895) observed "the various maltases that undoubtedly exist should be termed corn, yeast, etc. maltases depending on their origin", it is the exception rather than the rule to find molecular or enzymic identity

between functionally similar enzymes from different species (Paul and Fottrell, 1961). Even within the same species differences in functionally similar enzymes had been noted as early as 1937 when Bodansky by means of bile acid inhibition showed that the alkaline phosphatases of human intestine and bone were not identical. Abul-Fadl and King (1949) showed that in the same tissue, the human erythrocyte, there were two acid phosphatases which differed in pH optima and stability and in the ultimate case, that of a crystalline enzyme, ox-heart lactate dehydrogenase, Meister (1950) noted that it was composed of two protein fractions both of which possessed lactate dehydrogenase activity (Neilands, 1952). While because of these and other similar observations, the existence of functionally similar but distinguishable enzymes had been superficially accepted it was not until the demonstration by Wieland and Pfleiderer (1957) of the presence in a single tissue of several physically-separable lactate dehydrogenases that the phenomenon was generally accepted. This appreciation of the existence of multiple molecular forms of enzymes has provided a source for research which has spilled over into the fields of protein chemistry, physiology, pharmacology, genetics, evolutionary and developmental biology.

Obviously the many multiple forms of enzymes which have been described, even the few mentioned above, are of different types. The alkaline phosphatases would appear to be distinctly different proteins which are tissue specific within a species. Acid phosphatases are probably similar but there is also heterogeneity within tissues as Abul-Fadl and King

(1949) showed. Hopkinson, Spencer and Harris (1963, 1964) later demonstrated that the patterns of human red cell acid phosphatases separated electrophoretically are genetically determined and have recognised six phenotypes in European populations. Following the work of Vessell and Bearn (1957, 1958), Wieme (1959) and Wróblewski and Gregory (1960) who demonstrated the existence of five forms of human lactate dehydrogenase, patterns of which were tissue specific, the studies of Appella and Markert (1961) Cahn, Kaplan, Levine and Zwilling (1962), and Markert (1963, 1968) showed that these enzymes were tetramers composed of two different polypeptide subunits.

Nomenclature

Markert and Møller (1959) proposed the name isozyme, or isoenzyme (Wróblewski and Gregory, 1960), for the "different molecular forms in which proteins may exist with the same enzymatic specificity". This was meant to define the forms of lactate dehydrogenase since Markert and Møller found that "all of the lactate dehydrogenase isozymes were alike in the relative catalytic efficiency displayed towards different substrates". However this judgement was only based on qualitative evidence using hydroxyacid substrates to visualise the positions of the lactate dehydrogenase following electrophoresis. It had already been shown by Hess (1958) that the different forms showed differences in affinity for pyruvate, in optimum pH and in sulphite inhibition, while Hill (1958) had reported differences in heat stability. This definition did not therefore categorise the types of multiple enzyme forms which were to be called

isoenzymes, except possibly to exclude that one for which it was specifically introduced.

Webb (1960) pointed out that there was probably a continuous range of cases and that any division of this range must be completely arbitrary. He further suggested that the fundamental distinction between those proteins which are recognised as different enzymes and those as isoenzymes might be that the former were synthesised under the control of different genes and the latter were modifications of a single gene product. However neither this definition nor that of Augustinsson (1961) who suggested that the term isoenzyme be "restricted to enzymes the molecular structures of which differ only in those parts of the molecule that are not directly involved in the enzymatic reaction" appear capable of practical implementation in our present state of knowledge.

More practicable was the suggestion of Wieland and Pfleiderer (1962) that the name heteroenzyme be given to proteins of different origin which had the same biological action but differed in physical, chemical and biochemical properties and that the term isoenzyme or multiple form be given to enzymes from the same source with very similar but distinguishable proteins. This system would categorise the alkaline phosphatases as heteroenzymes and the red cell acid phosphatases and lactate dehydrogenases as isoenzymes.

The Standing Committee on Enzymes of the International Union of Biochemistry (Webb, 1964) then put forward the recommendation that "multiple enzyme forms in a single species should be known as isoenzymes, although since either form

is readily intelligible, this recommendation is not to be interpreted as excluding the use of 'isozyme' if any individual author prefers it". This retrograde recommendation classified all multiple forms as isoenzymes and the situation concerning definition and nomenclature became so confused that King (1965) noted that unless the International Union of Biochemistry clarified and defined the new concepts of different molecular forms of enzymes, many of the terms would soon cease to have any meaning.

Recently further recommendations have been published (International Union of Biochemistry, 1972) dealing specifically with isoenzymes and genetic variants. Basically two recommendations were made; that "the term 'multiple forms of an enzyme' should be used as a broad term covering all proteins possessing the same enzymatic activity and occurring naturally in a single species" and that "the term 'isoenzyme' or 'isozyme' should apply only to those multiple forms of enzymes arising from genetically determined differences in primary structures and not to those derived by modification of the same primary sequence". Of the seven groups of multiple forms listed, only three, those due to genetically independent proteins as alkaline phosphatases, heteropolymers of two or more polypeptide chains, non-covalently bound such as lactate dehydrogenase and allelic variants such as red cell acid phosphatases are isoenzymes. Multiple forms derived from proteins conjugated with other groups, proteins derived from one polypeptide chain, protein polymers of a single subunit and proteins differing in conformation, that is allosterically modified, are not to be classed as isoenzymes.

It may be a moot point as to whether acetyl-cholinesterase and cholinesterase possess "the same enzymatic activity" to a lesser degree than placental and liver alkaline phosphatases but for the present undertaking they will be treated as distinctly separate entities. The cholinesterases synthesised at the first and second loci are certainly of the first category that of genetically independent isoenzymes while the variants at the first locus are obviously allelic isoenzymes. Of the fractions separated by electrophoresis the foetal cholinesterase probably differs only in its conjugation with sialic acid while the others most likely represent polymers of a single subunit and therefore these multiple forms are not classed as isoenzymes.

Methods of Characterisation

The means available for the recognition and differentiation of isoenzymes fall into four categories, physical, chemical, immunological and biochemical.

Physical Methods

Most of these techniques require extensive preparation and handling; preferably the enzyme should be in a high state of purity, ideally in crystalline form. Then determination of molecular weight by ultracentrifuge, potentiometric titration of acidic and basic groups, determination of solubility and isoelectric point, ultraviolet absorption, rotational dispersion and X-ray crystallography may be attempted. Column chromatography on ion-exchange resins or uncharged adsorbers and gel filtration are also available without obligatory preliminary purification. Human serum

cholinesterase has been purified 13,000 fold (Das and Liddell, 1970) but crystalline preparations of the enzyme have not yet been obtained. The data obtained from such means have been summarised earlier (page 3).

Electrophoresis has undoubtedly played the single most important role in the recognition and separation of multiple forms of enzymes. It has certain inherent advantages in that it has wide applicability, is rapid, achieves a high degree of resolution and is less likely to produce artifacts than some other methods. This technique has been successfully applied to the separation of the polymeric multiple forms of serum cholinesterase and the first and second loci isoenzymes (page 4 and 17). However, physical methods have not been so efficacious with regard to the allelic isoenzymes and conflicting reports have appeared concerning their electrophoretic and chromatographic separation. Kalow (1959) obtained no differentiation by fractional precipitation with ammonium sulphate nor by chromatography on DEAE cellulose. Attempts to separate the usual and atypical variants by means of electrophoresis were also unsuccessful (Kalow, 1959; Ecobichon and Kalow, 1963; Svensmark, 1963). However, Liddell, Lehmann, Davies and Sharih (1962) claimed to obtain complete separation of the usual and atypical variants by paper electrophoresis at pH 9.7 and partial separation of the fluoride-resistant isoenzyme (Liddell, Lehmann and Davies, 1963). Separation on DEAE-cellulose was also reported by Liddell and colleagues (1962). Again Hodgkin, Giblett, Levine, Baur, and Motulsky (1965) found no difference between sera of usual and atypical homozygotes by starch-gel

electrophoresis nor by immunoelectrophoresis.

Chemical Methods

Procedures in this category include determination of terminal and functional groups, of component groups either by two-dimensional paper chromatography or electrophoresis following proteolysis and indeed sequential analysis of the primary structure. In this field even moreso than in the last, enzyme purity is mandatory and accounts for the depressed statement of the International Union of Biochemistry (1972) that "with regard to structural criteria, it has long been the hope of many workers in the field that the inter-relationships of multiple forms might soon be delineated in chemical terms. However, such information is available only for a very few enzyme systems....."

Immunological Methods

Again these techniques require the enzyme proteins in individual states of high purity for the preparation of the antibody and it must be confessed that such methods are not as quantitative and reproducible as required where minor differences in protein structure are being investigated. This is indicated by the work of Hodgkin, Giblett, Levine, Baur and Motulsky (1965) who found that neutralisation of anti-human usual cholinesterase was obtained by similar amounts of serum from both usual and atypical homozygotes. The technique however has proved useful in establishing heterogeneity within the silent gene homozygote phenotype. Thus Hodgkin et al. (1965), Szeinberg, Pipano, Ostfeld and Eviator (1966) and Kattamis, Davies and Lehmann (1967)

found a lack of cross-reacting material in the sera of such individuals but Goedde, Gehring and Hoffmann (1965) described two similar serum specimens which neutralised the antibody to almost the same extent as normal serum. Presumably the latter cases represented structural alterations which resulted in loss of enzyme activity without loss of ability to react with normal enzyme antibodies.

Biochemical Methods

In contrast to the foregoing categories most of the biochemical criteria can be obtained without extensive preparatory work. Determinations of optimum pH, of substrate and cofactor affinities, of the effects of activators or inhibitors and of temperature coefficients are not unduly influenced by use of the enzyme in its natural milieu or in crude preparations. Vindication of this statement as far as human serum cholinesterase is concerned is given by the studies of Das and Liddell (1970) who purified the enzyme 13,000 fold. These workers found that the Michaelis constants with benzoylcholine, acetylthiocholine and butyrylthiocholine did not differ significantly for the untreated plasma and the purified enzyme. Similarly the inhibitory effects of various concentrations of dibucaine hydrochloride, sodium fluoride and the neostigmine analogue RO2-0683 (Hoffman La Roche) were recorded with 50 μ M benzoylcholine, 5mM acetylthiocholine and 7.5 mM butyrylthiocholine and identical results obtained with the purified enzyme and untreated plasma.

Further the findings of Kalow (1962) and Liddell, Newman and Brown (1963) indicate that in this particular

instance, studies on serum provide a valid picture of the cholinesterase present in other tissues. These workers obtained post-mortem specimens, in the studies of Liddell and colleagues, of liver, kidney, brain, ileum and skin from subjects homozygous and heterozygous for the usual and atypical serum variants and found the inhibition characteristics of the cholinesterase of the tissue extracts to be the same as serum. Doenicke, Gurtner, Kreutzberg, Remes, Spiess and Steinbereithner (1963) found, in a silent gene homozygote, that a liver biopsy specimen, like the serum, was devoid of cholinesterase activity. Evidently the mutant gene products are found throughout the body and their synthesis is controlled by the same genes in all tissues.

Of the biochemical criteria used in isoenzyme differentiation, that of the effect of pH, buffer and substrates has been extensively investigated for serum cholinesterase as indicated earlier (p. 7 et seq.). The use of differential inhibitors was the means by which the allelic isoenzymes were first distinguished and there is now a considerable literature on this aspect (p.16 et seq.). However little or no attention has been given to the effect of heat on cholinesterase kinetics although Kalow and Lindsay (1955) observed that "the effects of temperature on the rate of hydrolysis of benzoylcholine and procaine are almost identical, while the hydrolysis of acetylcholine is differently affected". Apparently no attempt has been made to investigate the possible differential effect of temperature on the enzymic kinetics of the allelic cholinesterases and Harris (1970) could conclude "it should be noted that in a number of properties,

for example thermostability and electrophoretic mobility the two forms of the enzyme do not apparently differ". Accordingly this appeared to be an area in which investigation was required and one which might provide insight into structural differences in the allelic cholinesterase isoenzymes.

1. (c) Effect of Heat on Isoenzymes.

(c) Effect of Heat on Isoenzymes.

Herbert (1944) was, if not the first, among the earliest workers to employ differences in thermolability as a means of differentiating enzyme species. She found that the acid phosphatase of prostate extract was irreversibly inactivated by incubation for one hour at 37°C at pH 7.4. At pH 4.8 the enzyme was more stable. The same result was obtained with prostatic extract added to human serum but the acid phosphatase of normal serum was not affected by this treatment. The pH of the sera during incubation was not recorded but was probably in excess of 8.0 since the pH of serum separated from cells rises from pH 7.4 to 8.0 in fifteen minutes at room temperature due to loss of carbon dioxide (King, 1965). In a later report (Herbert, 1946), the destruction of the acid phosphatase of prostatic extract in one hour at 37°C in a variety of buffers at pH 7.4 was confirmed but only a slight reduction in activity occurred at pH 4.8 under the same conditions.

This phenomenon was re-examined by Woodard (1951) who confirmed that the thermostability of prostatic acid phosphatase was pH-dependent. However she found that heating for one hour at 37°C at pH 8.0 or above, nearly, or quite completely, inactivated not only the acid phosphatase of the serum of patients with metastasising carcinoma of the prostate but also the acid phosphatase of normal female sera.

The difference in observations may have been the result of the different substrates employed; phenylphosphate by Herbert and B-glycerophosphate by Woodard. The latter

author indicated that she was aware that the behaviour of phosphatases varied considerably with different substrates but neither she nor Herbert appear to have examined the thermostability of the enzymes over a range of temperature. Herbert (1946) did note that during the five hour incubation proposed by Gutman and Gutman (1938) that although hydrolysis was constant at first there came a sharp fall-off in activity, sometimes before two hours, but always evident by three hours and progressing until hydrolysis almost ceased. Accordingly she reduced the reaction time to one hour. Again however she did not study the thermostability of the enzyme over a range of temperatures in the presence of substrate. In any case this possible means of differentiating the tissue-specific acid phosphatases does not appear to have been generally accepted and employed.

Indeed no further use of the phenomenon was apparently made in clinical enzymology until Wróblewski and Gregory (1961) utilised the differences in heat stability of the human lactate dehydrogenases earlier noted by Hill (1958). Wróblewski and Gregory showed that the electrophoretically-fastest fractions which predominate in heart muscle were unaffected by heating for 30 minutes at 57°C in the presence of reduced nicotinamide-adenine dinucleotide (NADH) which increased heat stability. A scheme for determining the relative amounts of isoenzymes present in serum was proposed. This consisted of adding 0.2 ml NADH solution (2.5 mg/ml) to 2 ml of serum, dividing into three aliquots which were then incubated for 30 minutes at bench temperature (A), 57°C (B) and 65°C (C) prior to assay of lactate dehydrogenase

activities. Total serum lactate dehydrogenase activity was given by specimen (A) the heat-labile fraction (LDH5) by (A) - (B), the heat-stable LDH1 by specimen (C) and intermediate fractions (LDH2, LDH3 and LDH4) by (B) - (C). A good correlation with electrophoretic separation was found in a wide variety of disease states by means of this heat stability test which however tended to give relatively high values for LDH5. This difference was attributed to inactivation of LDH5 by the heat generated during electrophoresis.

Independently, Strandjord and Clayson (1961) found that heating liver homogenates for 30 minutes at 65°C resulted in a loss of 99.5% of lactate dehydrogenase activity but only 47% of heart homogenate activity was destroyed under these conditions. They suggested measurement of the heat-stable lactate dehydrogenase in serum as a diagnostic index of myocardial infarction. In patients with congestive heart failure, hepatitis and after surgical operation the heat-stable lactate dehydrogenase activity was the same as in normal sera and this was confirmed and expanded in a later report (Strandjord, Clayson and Freier, 1963).

In the interval Van Der Helm, Zondag, Hartog and Van Der Kooi (1962) demonstrated that following heating for thirty minutes at 56°C, LDH3, LDH4 and LDH5 were no longer demonstrable by electrophoresis and the scheme of Wróblewski and Gregory (1961) was simplified by Bell (1963) and Latner and Skillen (1963) to heating at 60°C for one hour. The latter workers found that the "heat stability index", that is, the ratio of activities of the heated and unheated samples,

was greater than 0.5 in myocardial infarction, consistently less than this in other diseases and in health was less than 0.3. This last figure is difficult to reconcile with the known isoenzyme composition of normal sera and was not confirmed personally (King, 1966).

Even the simplified procedures have not enjoyed a widespread use in routine clinical practice mainly because of the lengthy periods of denaturation. Employment of a sub-optimal concentration of 2-oxobutyrate in the assay of "alpha-hydroxybutyrate dehydrogenase" (Rosalki and Wilkinson, 1960) has proved more acceptable, albeit possibly with less vindication of its diagnostic value (Ellis and Goldberg, 1971; King, 1972).

The relative stabilities to heat displayed by the isoenzymes of lactate dehydrogenase are almost mirrored by their cold stability. Zondag (1963) demonstrated that the LDH4 and LDH5 of liver extract in barbital buffer of pH 7.9 were destroyed overnight at -20°C . This occurred in the buffered extract only, was not evident in the tissue alone at -20°C and could be prevented in the buffered homogenate by addition of NAD. This addition also appeared to increase the speed of anodal migration of LDH1 and LDH2. The lability at low temperatures of the slower-moving fractions was confirmed by Kreutzer and Ferris (1964) who reported however that LDH4 was less stable than LDH5 under these conditions.

Differences in the heat-sensitivity of alkaline phosphatases were first reported by Moss and King (1962), who recorded, without further discussion, that at 55°C and

pH 7.0, the times required to reduce the activities of bone, kidney, intestine and liver isoenzymes to half the initial level were 8, 12, 15 and 18 minutes respectively. The remarkable stability to heat of placental alkaline phosphatase was later demonstrated by Neale, Clubb, Hotchkis and Posen (1965). The activity of this isoenzyme, which predominates in serum in the third trimester of pregnancy (McMaster, Tennant, Clubb, Neale and Posen, 1964) is not altered by heating for 30 minutes at 70°C in the presence of 10^{-2} M magnesium ions. The presence of the metal ion activator was not necessary with fresh placental homogenate or with serum enriched with placental enzyme.

The studies of Posen, Neale and Clubb (1965) to determine the source of origin by the heat lability of the alkaline phosphatase indicated that the heat sensitivity of each isoenzyme remained characteristic and independent of the influence of others in the mixture. They were able, by means of heating for 30 minutes at 56°C, to distinguish between "osteoblastic" and "hepatic" sera and to conclude that normal serum behaved like a mixture containing 50 to 75% of the skeletal isoenzyme. Further, on the observed differences between bile and liver isoenzymes they concluded that the serum alkaline phosphatase from patients with hepatic disorders behaves like a mixture, predominantly of the bile enzyme. Fishman and Ghosh (1967) confirmed most of this work but found that since the heat inactivation of the intestinal enzyme (50 - 65%) was similar to that of the liver (50 - 75%) it was not possible to determine the tissue source of the serum alkaline phosphatase by heat

sensitivity alone. An indication of the amount of intestinal enzyme present by means of L-phenylalanine inhibition was also deemed necessary.

In all of these studies it was the permanent irreversible effect of heat on the enzyme protein which had been examined, and while this was followed over a wide range of temperatures in some, it was only the total loss of activity by one species following prolonged incubation that was sought. Indeed the progressive effect of heat upon enzyme activity in the presence of substrate, apart from establishing temperature correction factors had apparently not been studied in clinical enzymology. Certainly it had not been examined with a view to possible usefulness in differentiating enzyme species.

Interest in this field was first awakened by the need to determine temperature correction factors for a number of serum enzymes (King, 1965). This work revealed that the temperature-activity curves showed no abrupt point of denaturation and while one temperature might denature one enzyme and not another, this was only a particular facet of an individual, characteristic temperature-activity relationship. It was obvious that where there were differences in the so-called "optimum temperature", that beyond which inactivation played a major role, then there were equally discernible differences at lower temperatures. Thus it was found that the ratios of activities at 37°C and 25°C were 2.78 for caeruloplasmin, 2.13 for aspartate transaminase and 1.88 for cholinesterase. It could be argued that these represent different categories of enzymes but this is not

a valid point. As it is even in the same class, say the oxido-reductases, differences as wide were found with values of 2.78 for isocitrate dehydrogenase; 2.5 for malate dehydrogenase; and 1.89 for 6-phosphogluconate dehydrogenase.

Early studies on heat denaturation at 60°C with lactate dehydrogenase and malate dehydrogenase in the sera of patients suffering from a wide range of diseases had indicated that clinically useful information could be derived from the differing isoenzyme responses to heat (King, 1966). On the practical side however the delays during the pre-assay denaturation process militated against its employment in routine clinical laboratories. A study of the temperature-activity relationship of lactate dehydrogenase in human serum was then undertaken and while distinct differences were noted depending upon the isoenzyme composition there was invariably present in varying proportions at least four of the five isoenzymes as revealed by agar-gel electrophoresis (Wieme, 1959). The same applied to tissue extracts and facilities for isolating the isoenzymes of human lactate dehydrogenase were not available at that time. The same impediments applied to the "tissue-specific" isoenzymes of alkaline phosphatase. The obvious solution to this was the study of an enzyme, the molecular species of which were already completely separated in human sera, and in this respect the genetically-determined isoenzymes of cholinesterase appeared to be a perfect example. In homozygous individuals only one enzyme variant is present. Further cholinesterase is clinically labelled as a "serum-specific" enzyme and as such evinces a relatively high

activity precluding the need for preliminary purification.

1. (d) Aims of the study.

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The scheme of work envisaged was an investigation into methods of differentiating the human serum cholinesterase variants and an examination of these as a means of assessing molecular differences. An emphasis was to be placed on the effect of heat on the enzyme kinetics.

It was considered that studies with various inhibitors and substrates might reveal further phenotypes and possibly give some indication of the structural differences. On a more practical plane it was hoped to elaborate simple screening procedures for suxamethonium-sensitive individuals and to gain enlightenment on the anomalies between biochemical parameters and physiological observations.

2. Benzoylcholine Hydrolysis.

(a) Temperature studies.

Benzoylcholine has certain recommendations as a substrate for studies on cholinesterase. First, it has the advantage of specificity (Mendel and Rudney, 1943) and therefore although it was proposed only to examine human serum, even a degree of haemolysis would not invalidate results. Secondly, benzoylcholine has an absorption maximum at 232 nm much greater than its hydrolysis products which absorb maximally at 224 nm. The rate of hydrolysis can therefore be readily monitored in an ultraviolet spectrophotometer. The validity of an initial reaction rate assay compared to other types of enzyme activity determination is well established. Finally, benzoylcholine has proved to be a particularly useful substrate in studying the allelic variants of human cholinesterase (Kalow and Staron, 1957; Harris and Whittaker, 1962). Indeed it was with this substrate in the assay procedure of Kalow and Lindsay (1955) that the means of identifying these variants was discovered by dibucaine inhibition (Kalow and Genest, 1957). Almost as a matter of custom since then, the same assay procedure has been employed in investigations with other inhibitors in differentiating the human cholinesterase allelic isoenzymes.

Kalow and Lindsay (1955) did not follow the substrate hydrolysis at 232 nm but at 240 nm. The difference in absorptions between benzoylcholine and its hydrolysis products, mainly benzoate, is greatest at 235 nm but this is reduced by less than 10% at 240 nm. At the lower wavelength the dilute serum itself has an absorption of 1.2 but at 240 nm this is reduced to 0.5. This latter

wavelength was obviously chosen as giving an acceptable initial absorption while retaining adequate sensitivity in following the hydrolysis of benzoylcholine. A 50 μ M concentration of benzoylcholine in the final reaction mixture was employed at pH 7.4 given by a $\frac{M}{15}$ Sørensen phosphate buffer. These workers were obviously aware that these conditions did not give maximal activities but, as with the choice of wavelength, were optimal compromises. This knowledge was explicit in later reports which gave a ratio of 5.5 between the Michaelis-Menten constants for the atypical and usual enzymes and of 0.5 for the V_{max} ratio (Davies, Marton and Kalow, 1960) thereby indicating that the substrate concentration for maximal activity varied with the cholinesterase phenotype.

Preliminary experiments with the limited phenotype sera available at the beginning of this project indicated that a 50 μ M benzoylcholine concentration was indeed optimal for the usual enzyme at 25°C. The atypical enzyme required a substrate concentration of about 125 μ M for saturation. However since inhibition by excess substrate was found with all variants tested and most acutely with the usual enzyme, it was obvious that unless the substrate concentration were to be varied for each phenotype then that optimal for the usual enzyme was the best compromise.

Tentative investigations into the effect of pH on benzoylcholine hydrolysis were generally in agreement with the findings of Kalow (1964). The optimum pH varies with the type and ionic strength of the buffer, with the substrate concentration and also with the enzyme variant. With a

50 μ M benzoylcholine concentration and $\frac{M}{15}$ Sørensen phosphate buffer the usual enzyme has an optimum pH about 9.0, the atypical about 8.5. At a 100 μ M substrate concentration the atypical pH optimum was slightly increased towards that of the usual enzyme at its optimal substrate concentration. At these hydrogen ion concentrations however the spontaneous hydrolysis of the substrate was no longer negligible and certainly not at higher temperatures. This factor presumably had made for the original choice of pH 7.4.

As it was, the proposed scheme of study did not envisage elaborating new assay conditions if those already available and well-tested were adequate. Obviously the kinetics of cholinesterase are very complex and any new set of assay conditions would be difficult to justify as superior to those originally set out by Kalow and Lindsay (1955). Even if this could have been done the knowledge with regard to phenotyping accumulated over the intervening years would be void, and it would be an empty exercise to repeat this with the new assay conditions. Accordingly the assay procedure of Kalow and Lindsay (1955) which had been found to give remarkably reproducible results was adopted without modification for temperature studies. The negligible variation with temperature in the difference in molar absorptivities of benzoylcholine and benzoate at pH 7.4 was a bonus since this meant that the rate of change in absorption (ΔA) was directly and constantly related to enzyme activity at all temperatures studied.

Instrumentation, Methods and Materials

Initially, estimations were carried out on a Unicam SP 800 recording spectrophotometer with scale expansion to a Servoscribe recorder. Temperature control was obtained by circulating water from a Shandon water-bath through the jacketed cuvette-holder. Cooling was obtained by adding ice chips to the water-bath. Later, a Unicam SP 8000 recording spectrophotometer with Unicam AR 25 linear recorder and with temperature control given by a Tecam water-bath and Grant Instruments dip-cooler was employed. In the final stages, studies were performed on a Unicam SP 1800 recording spectrophotometer with Unicam AR 25 linear recorder, constant temperature being assured by a circulating Heto water-bath and Grant Instruments dip-cooler. This last group of instrumentation was provided by the Medical Research Council under grant G971/343/C and acknowledgment of this generous support is gratefully recorded.

With all these instruments the temperatures of the water-bath and of the cuvette contents were correlated over the temperature range 15°C to 60°C by means of a standardised mercury thermometer and Wayne Kerr contact thermometer respectively. The difference between the bath and cuvette temperatures increased with temperature deviation from the ambient as did the time required for equilibration. Temperature control was improved by the use of Techne polypropylene spheres floating in the water-bath.

Oxford pipettes were used for sampling or dispensing

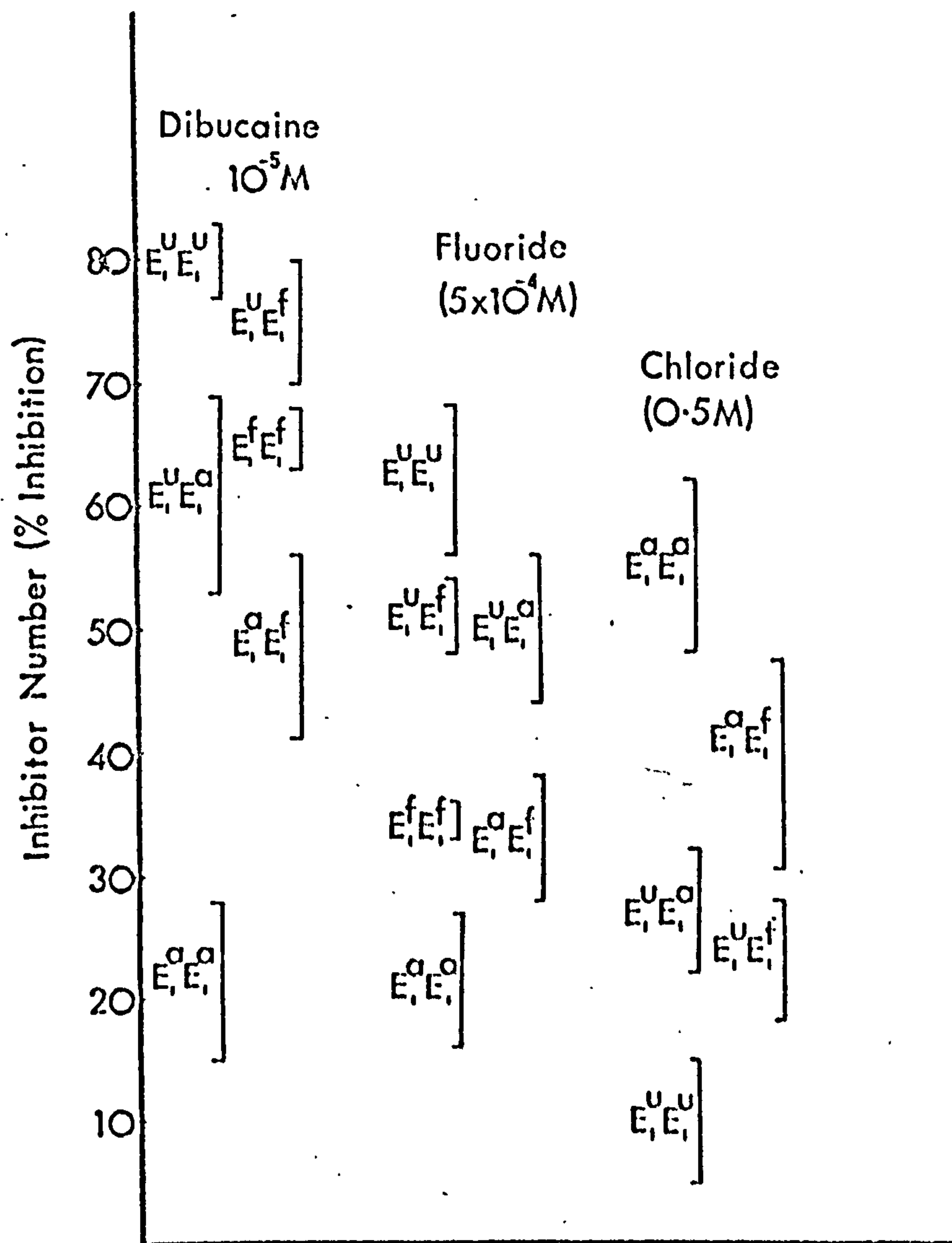


Figure 2. Phenotype ranges of inhibitor numbers.

These inhibitor numbers or percentage inhibitions refer to the assay procedure of Kalow and Lindsay (1955) with a 50 μM benzoylcholine substrate in phosphate buffer at pH 7.4.

The ranges refer to 25°C from earlier personal experience and to 26.5°C from Whittaker (1968b) and Lehmann and Liddell (1969).

all volumes less than 2 millilitres.

Serum cholinesterase activity was estimated by the procedure of Kalow and Lindsay (1955), modified by King (1965) and results were expressed in nanomoles of benzoylcholine hydrolysed per minute by one millilitre of serum, that is, mU/ml (International Union of Biochemistry, 1961; 1965). Normal values obtained by this method range from 620 to 1370 mU/ml at 25°C (King, 1965). For cholinesterase phenotyping, activity was simultaneously and separately assayed in the presence of 10 µM dibucaine (Kalow and Genest, 1957), 50 µM sodium fluoride (Harris and Whittaker, 1961) and 500 mM sodium chloride (Whittaker, 1968b). All phenotyping assays were performed at 25°C. Figure 2 indicates the ranges of inhibition by dibucaine, fluoride and chloride, culled from the literature and personal experience and which were used to phenotype serum specimens.

Reagents

Benzoylcholine, sodium fluoride, sodium chloride and orthophosphate salts were obtained from BDH Chemicals Ltd. and the cinchocaine hydrochloride (dibucaine hydrochloride) from Ciba.

- (i) 67 mM. Sørensen phosphate buffer, pH 7.4. Dissolve 19.19 g disodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) and 3.48 g potassium dihydrogen phosphate (KH_2PO_4) in water and make to 2 litres.
- (ii) 200 µM substrate solution. Dissolve 48.8 mg benzoylcholine chloride in 1 litre of water.
- (iii) 40 µM dibucaine solution. Dissolve 15.2 mg cinchocaine hydrochloride in 1 litre of water.

(iv) 200 μ M sodium fluoride solution. Dissolve 8.4 mg sodium fluoride (AR) in 1 litre of water.

(v) 2M sodium chloride solution. Dissolve 117 g sodium chloride (AR) in water and make to 1 litre.

Procedure

A 1 in 200 dilution of serum in buffer was prepared and placed in the blank cuvettes. A further 100 μ l of serum was diluted to 10 ml with phosphate buffer and 2 ml of this dilution added to each of the four test cuvettes. To the first cuvette, that giving the cholinesterase activity, was added 1 ml of water and to the other three cuvettes 1 ml of the respective inhibitor solution. Time was allowed for temperature equilibration, then 1 ml of substrate solution was added to each cuvette, the contents thoroughly mixed with a plastic paddle and the reaction rates recorded at 240 nm for a minimum of five minutes. From this the decrease in absorption per minute (ΔA) was obtained.

Using the micromolar extinction coefficient for benzoylcholine of 6.6 at 240 nm (Kalow and Lindsay, 1955) then in a 4 ml reaction volume with a light path of 10 mm activity is given by:-

$$\frac{4}{6.6} \Delta A = \mu \text{ moles benzoylcholine hydrolysed per min per } 20 \mu\text{l serum}$$

$$\frac{4}{6.6} \cdot \frac{1000}{20} \cdot 1000 \Delta A = n \text{ moles/min/ml}$$

$$\text{or} \quad 30300 \Delta A = \text{mU/ml}$$

It is not necessary to calculate the activities in the presence of the inhibitors and the inhibitor numbers

(percentage inhibition) were calculated as:-

$$100 \left(1 - \frac{\text{change in absorbance of inhibited assay}}{\text{change in absorbance of uninhibited assay}} \right)$$

In all of these studies the substrate solution and pipette tips were always maintained at the bath temperature which, below ambient was lower, and above ambient higher than the cuvette contents. Opening the cuvette compartment causes a slight change in the cell-housing temperature in an opposite direction so that the two effects have a tendency to cancel each other. Certainly after 30-60 seconds no consistent, significant variation in the reaction rates with time was evident within the limits imposed by substrate saturation.

It was also established that the activity and the response to inhibitors of serum diluted in phosphate buffer did not alter significantly during eight hours at bench temperatures. All temperature variation experiments, usually from 15°C to 60°C in steps of 5°C were undertaken in one run which usually took from six to eight hours. For these temperature studies therefore 1 ml of serum was diluted to 100 ml with phosphate buffer. This was then employed throughout the experiment and reduced errors due to preparing fresh dilutions for each temperature.

The enzyme source for these studies was sera from normal, healthy volunteers, from hospital patients with a wide variety of pathologies, from patients who had exhibited prolonged apnoeic responses to suxamethonium and from their relatives. A service was instituted at the Department of Biochemistry, Royal Infirmary, Glasgow which offered to phenotype any serum specimen referred and also offered

cholinesterase phenotyping to close relatives of those confirmed as sensitive by inhibition studies. Sensitivity cards (Telfer, McDonald and Dinwoodie, 1964) are issued to all individuals of the four phenotypes invariably at risk, that is, $E_1^a E_1^a$, $E_1^a E_1^f$, $E_1^f E_1^f$ and $E_1^S E_1^S$.

As a result of the widespread use which has been made of this service, an adequate supply of material has been available and individuals of all the ten generally-recognised genotypes have been discovered, some the first recorded in Scotland. Table 1 indicates the generally-accepted genotypes at the first locus, the nomenclature employed (Motulsky, 1964) and the frequency of occurrence in the population (Lehmann and Liddell, 1969).

PHENOTYPE	GENOTYPE	FREQUENCY
Usual Homozygote	$E_1^U E_1^U$	95 in 100
	$E_1^U E_1^S$	1 in 190
Usual/Fluoride-resistant Heterozygote	$E_1^U E_1^f$	1 in 280
Usual/Atypical Heterozygote	$E_1^U E_1^a$	1 in 26
Fluoride-resistant Homozygote	$E_1^f E_1^f$	1 in 300,000
	$E_1^f E_1^S$	1 in 200,000
Atypical/Fluoride-resistant Heterozygote	$E_1^a E_1^f$	1 in 29,000
Atypical Homozygote	$E_1^a E_1^a$	1 in 2,800
	$E_1^a E_1^S$	1 in 20,000
Silent Homozygote	$E_1^S E_1^S$	1 in 140,000

Table 1. Frequency of occurrence of the cholinesterase phenotypes.

Experimental Results

As indicated earlier (page 35) differentiation of isoenzymes by heat had only been attempted where there were differences in the irreversible denaturation of the molecular species. To determine whether such differences exist between the cholinesterase variants, serum specimens of the three usual/atypical genotypes were incubated for various periods at temperatures from 25°C to 60°C. The samples were then assayed at 25°C for cholinesterase activity. Below 50°C there was no evidence of denaturation in any sample for incubation periods up to one hour. Table 2 shows some typical results obtained from this series of experiments which indicated that there was no substantial difference in the irreversible thermal denaturation of the cholinesterase variants studied. That the change was irreversible was confirmed by re-assay of the specimens four to six hours after original removal of the heated aliquot and dilution in buffer (Table 2).

These denaturation experiments were carried out at the 50 μ M benzoylcholine concentration of Kalow and Lindsay (1955) which preliminary studies had indicated was substrate-saturating at 25°C for usual homozygote sera. The preliminary studies had also confirmed the findings of Davies, Marton and Kalow (1960) that heterozygotes had higher optimal substrate concentrations. Obviously these optimal concentrations would increase with temperature but no cognisance of this or of change in the optimal pH with temperature was taken in the next series of experiments. In this study sera from patients who had undergone prolonged paralysis

following suxamethonium administration and from members of their families were assayed at increasing temperatures as indicated under Methods. Preliminary studies (King, 1967) had shown striking differences in the effect of heat on the enzyme reaction rate and these are confirmed and extended in the following family studies (King and Dixon, 1969; King, McQueen and Morgan, 1971).

Table 2.

<u>Heat Denaturation of Serum Cholinesterase</u>							
Temperature	(minutes)	E_1^u	E_1^u (J.B.)	E_1^u	E_1^a (M.G.)	E_1^a	E_1^a (J.L.)
50°C	0		610 mU/ml		580 mU/ml		184 mU/ml
	5		98.5%		98.5%		97%
	30		93%		96.5%		91%
	60		75.5%		79.5%		73%
55°C	0		585 mU/ml		560 mU/ml		176 mU/ml
	5		65%		73%		76%
	30		12.5%		19%		29%
	60		1.5%		1.5%		3.5%
60°C	0		606 mU/ml		565 mU/ml		179 mU/ml
	5		3%		3.5%		10%
	30		1.5%		1.5%		6.5%
	60		1.5%		1.5%		3.5%
Repeat assays after 4 to 6 hours							
50°C	0		615 mU/ml		575 mU/ml		173 mU/ml
	5		95%		95%		96.5%
	30		84%		90%		88%
	60		70%		76%		72%
55°C	0		615 mU/ml		585 mU/ml		186 mU/ml
	5		68%		72%		77.5%
	30		17%		19%		29%
	60		3.5%		-		10%

Case 1

The patient (A) exhibited prolonged apnoea after suxamethonium was given during an obstetric procedure prior to delivery. Assay of her serum yielded values of 395 mU/ml for cholinesterase activity, 21 for dibucaine number (DN) and 17 for fluoride number (FN). She was accordingly classified as phenotype $E_1^a E_1^a$, homozygous for the atypical enzyme. Later figures for the son (B) were; serum cholinesterase 700 mU/ml, DN = 58, FN = 46 indicating a genotype $E_1^u E_1^a$ while the father (C) was a usual homozygote, $E_1^u E_1^u$ with cholinesterase activity of 920 mU/ml, DN = 78 and FN = 66. Cord blood serum from a second child, a daughter (D) permitted classification as an atypical heterozygote with cholinesterase activity 470 mU/ml, DN = 61 and FN = 50.

The cholinesterase temperature/activity relationship of this family are shown in Figure 3.

Case 2

The brother (E) of a suxamethonium-sensitive patient was found to have a serum cholinesterase activity of 475 mU/ml, dibucaine number 51, fluoride number 46 and was therefore classed as an atypical heterozygote, $E_1^u E_1^a$. Of his two sons, one (F) was apparently a usual homozygote with DN = 77 and FN = 62 but with a low serum cholinesterase activity of 370 mU/ml while the other (G) exhibited an even lower activity of 220 mU/ml with DN = 29 and FN = 27. These latter figures suggested an atypical homozygote but investigation of the mother's serum gave values of 555 mU/ml for cholinesterase activity and 80 and 57 for percentage

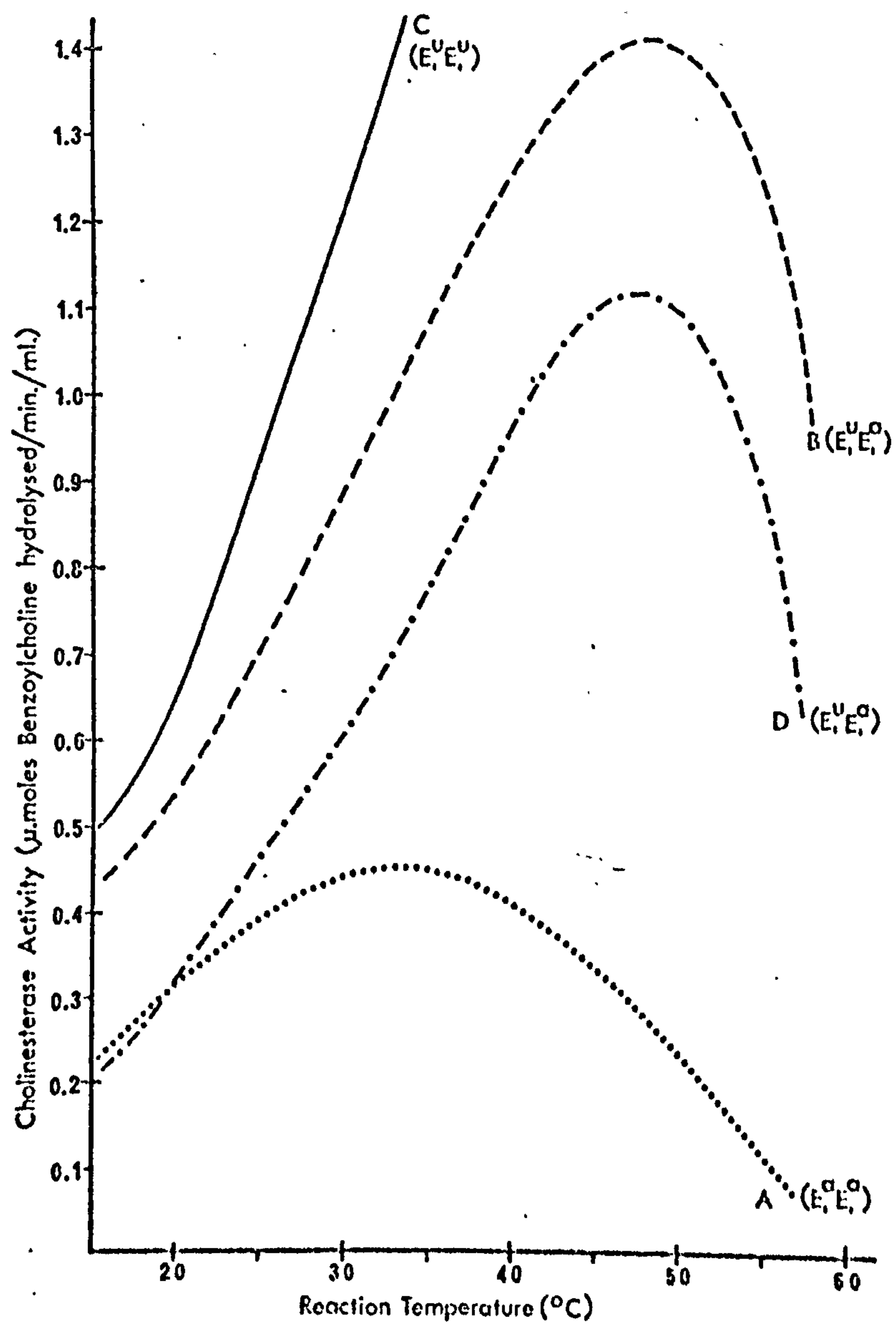


Figure 3. Cholinesterase temperature/activity curves of designated family members of Case 1 (page 49).

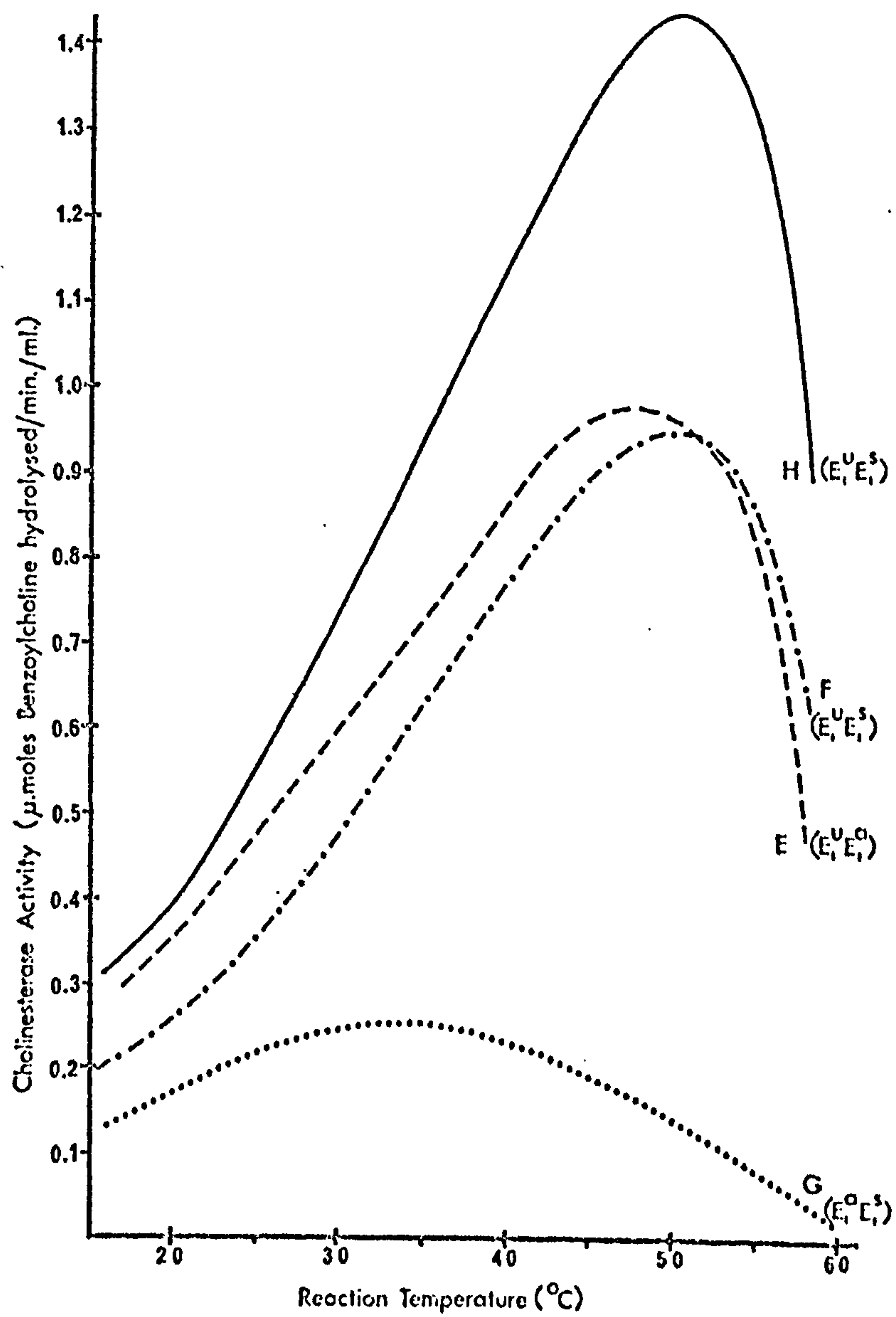


Figure 4. Cholinesterase temperature/activity curves of designated family members of Case 2 (pages 49, 50).

dibucaine and fluoride inhibition respectively. The presence of a silent gene in the mother (H) was therefore evident, assumed in the son (F) and both were classified as genotype, $E_1^u E_1^s$. The second son was obviously an atypical/silent heterozygote, $E_1^a E_1^s$.

The variation of serum cholinesterase activity with temperature in this family is illustrated in Figure 4.

Case 3

Following suxamethonium injection during dental anaesthesia patient (J) was apnoeic for 35 minutes. Serum assays indicated that he was heterozygous for both dibucaine - and fluoride-resistant genes, $E_1^a E_1^f$ with cholinesterase activity of 305 mU/ml, DN = 42 and FN = 34. Both his daughter (K) and a grand-daughter (L) were classified as atypical heterozygotes, $E_1^u E_1^a$, with cholinesterase activities of 640 mU/ml and 685 mU/ml, dibucaine numbers of 65 and 67 and fluoride numbers of 55 and 52 respectively. Figure 5 illustrates the temperature/activity curves for serum cholinesterase in the three generations of this family.

Case 4

The patient (M) suffered prolonged paralysis following suxamethonium administration prior to external céphalic version of her second child. Assay of her serum at this time yielded anomalous figures (page 124) but later results indicated a phenotype of $E_1^f E_1^f$ with cholinesterase activity of 480 mU/ml and dibucaine, fluoride and chloride numbers of 64, 36 and 28 respectively. The baby girl was found to

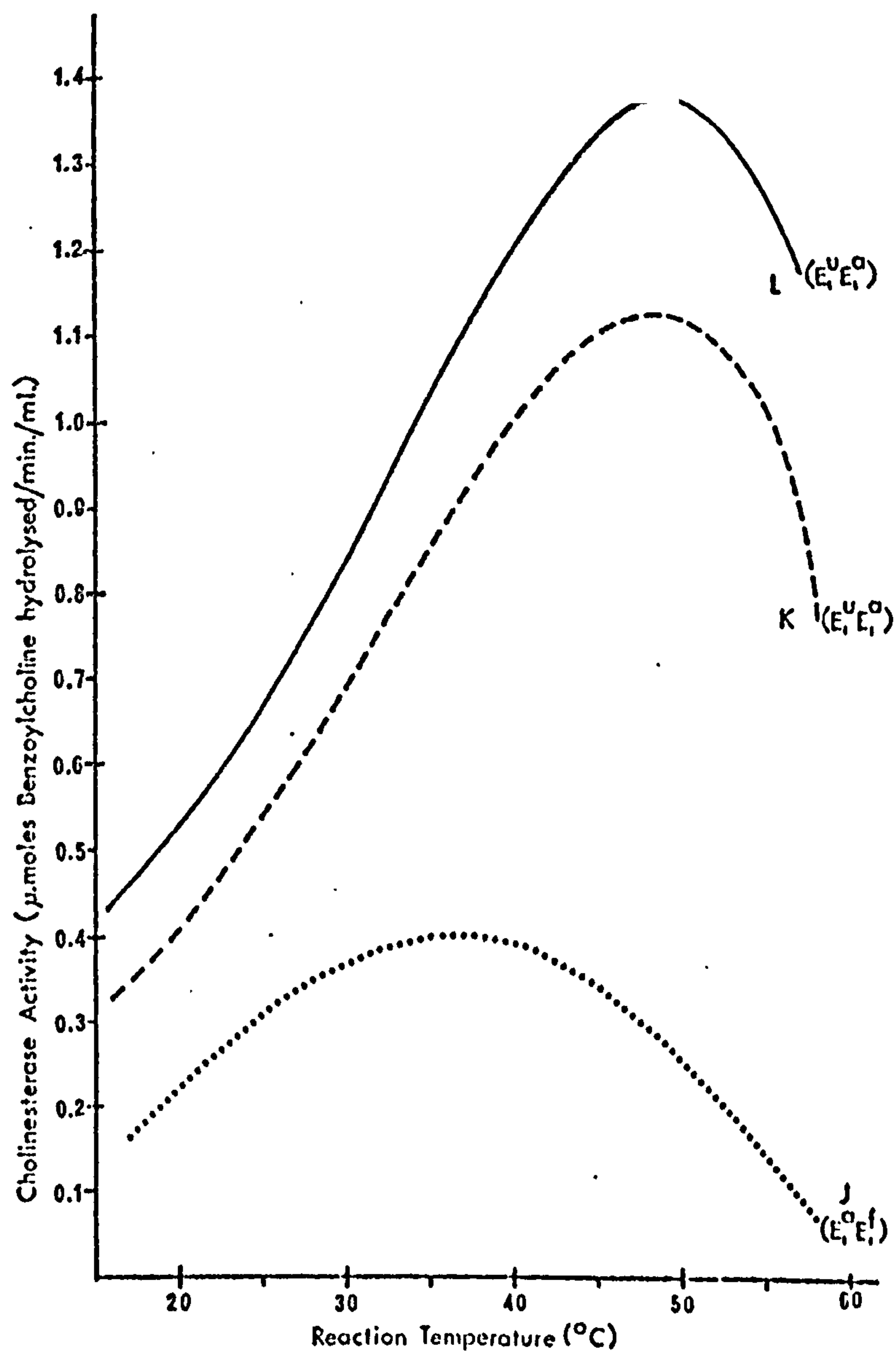


Figure 5. Cholinesterase temperature/activity curves of designated family members of Case 3 (page 50).

be genotype $E_1^u E_1^f$ as was the elder boy (O) whose serum cholinesterase activity was 1010 mU/ml with DN = 70, FN = 47 and Cl N = 19. Assay of the husband's serum (N) gave results for cholinesterase activity of 695 mU/ml and DN = 79, FN = 59 and Cl N = 10, a usual phenotype although the rather low activity in a healthy young adult might suggest the presence of a silent gene. Equally, of course, it was realised that the patient's phenotypic behaviour might mask a silent gene.

The patient's mother was deceased but her father (P) with dibucaine, fluoride and chloride numbers of 74, 52 and 18 respectively was classified as a usual/fluoride-resistant heterozygote, $E_1^u E_1^f$ as was paternal aunt. On examining the sera of the maternal uncle and aunt it was found that they were homozygous for the usual and fluoride-resistant genes respectively. The aunt (Q) had a serum cholinesterase activity of 78 mU/ml, DN = 67, FN = 39 and Cl N = 26, a second fluoride-resistant homozygote in the same family but in an earlier generation. This evidence for the presence of a fluoride-resistant gene in the maternal family made it unnecessary to postulate the presence of a silent gene and the cholinesterase activity of 1400 mU/ml in the maternal uncle made it unlikely. The temperature-activity relationships of the members of this family given letters in the text are shown in Figure 6. Details of the serum assays on all members studied are given in Table 3 and the cholinesterase phenotyping is illustrated in Figure 7.

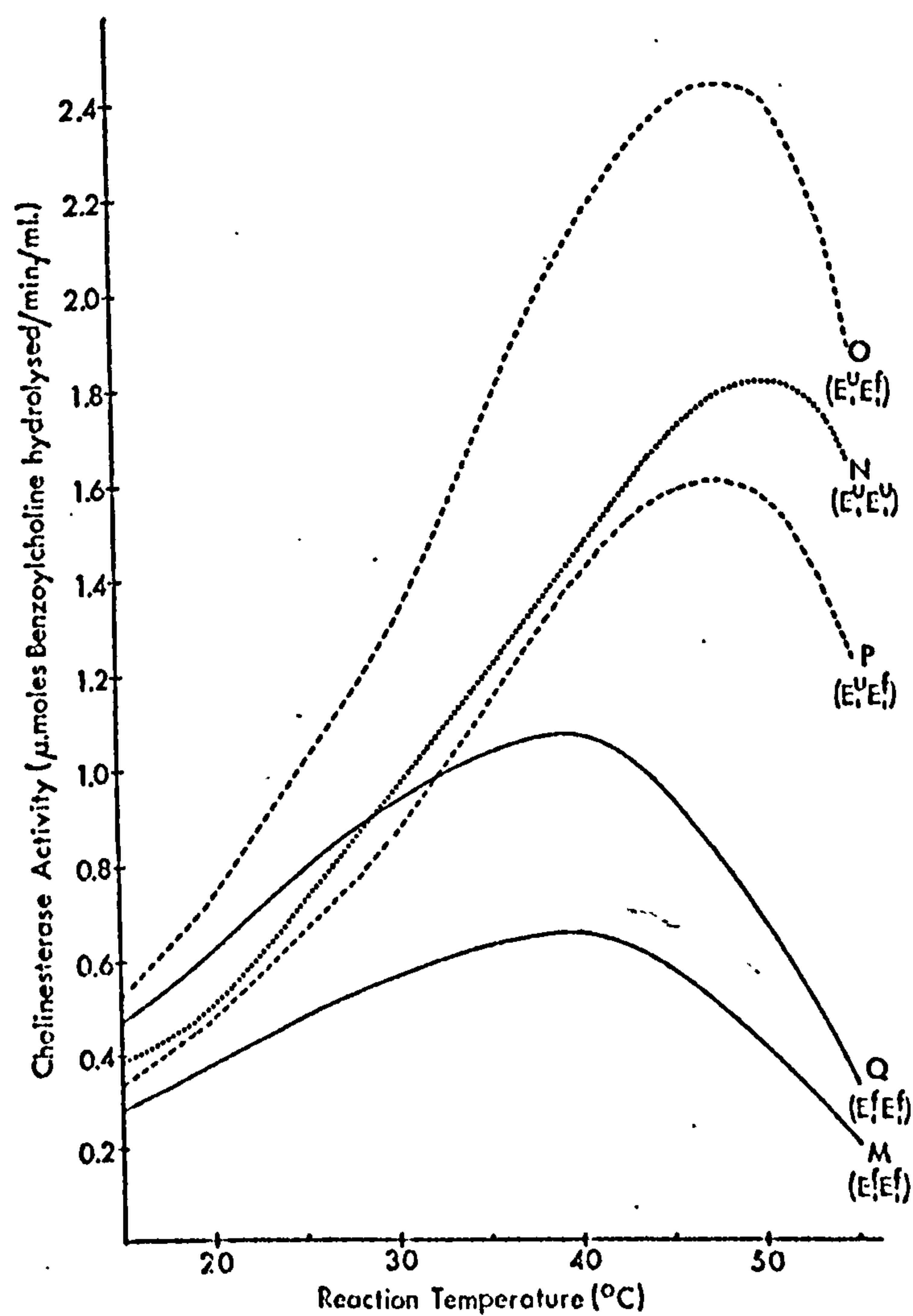


Figure 6. Cholinesterase temperature/activity curves of designated family members of Case 4 (pages 50, 51).

Table 3

Assay values for the family of Case 4

	Serum cholinesterase (mU/ml at 25°C)	Dibucaine Number	Fluoride Number	Chloride Number	Phenotype
Patient (M)	480	64	36	28	$E_1^f E_1^f$
Son (O)	1010	70	47	19	$E_1^u E_1^f$
Daughter	580	72	55	18	$E_1^u E_1^f$
Husband (N)	695	79	59	10	$E_1^u E_1^u$
Father (P)	645	74	52	18	$E_1^u E_1^f$
Paternal Uncle	1070	82	64	13	$E_1^u E_1^u$
Paternal Aunt	880	77	48	20	$E_1^u E_1^f$
Maternal Uncle	1400	81	59	16	$E_1^u E_1^u$
Maternal Aunt	780	67	39	26	$E_1^f E_1^f$

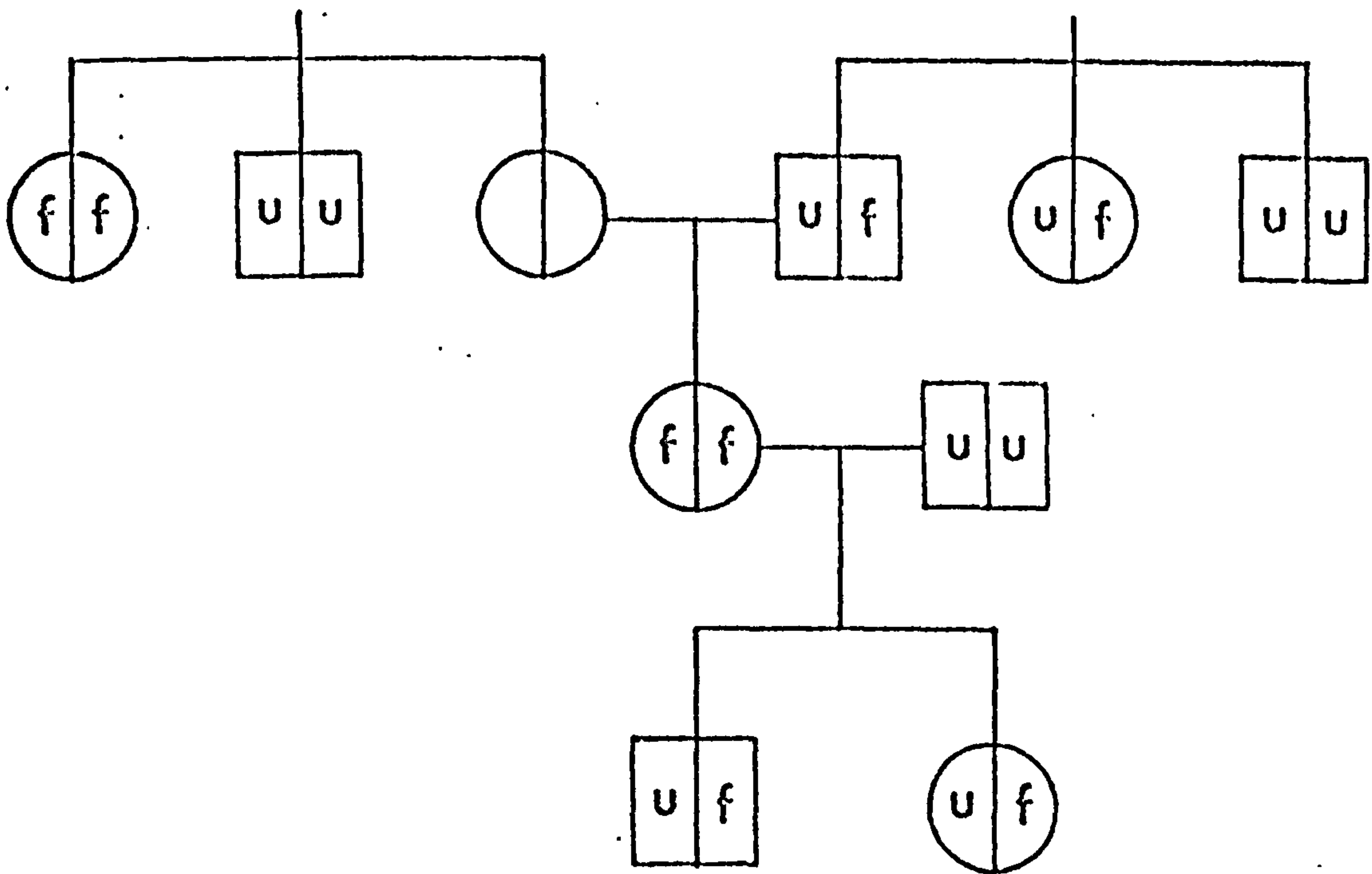


Figure 7. Phenotype relationship of Case 4 family (pages 50, 51).

Case 5

The patient (R) was a month-old baby boy. Serum assay gave a cholinesterase activity of 530 mU/ml, dibucaine number 52, fluoride number 29 and chloride number 39 consistent with a genotype $E_1^a E_1^f$. Cholinesterase phenotyping of the parents indicated that the father (S) was an atypical heterozygote with cholinesterase activity of 585 mU/ml, DN = 60, FN = 48 and Cl N = 23 while the mother (T) had a serum cholinesterase of 210 mU/ml with dibucaine, fluoride and chloride numbers of 62, 33 and 28 respectively. The inhibitor numbers suggested that she was a fluoride-resistant homozygote, $E_1^f E_1^f$, but assay of her parents' sera revealed that she was in fact a fluoride-resistant/silent heterozygote since her father (U) was genotype $E_1^u E_1^f$ and her mother (V) apparently a usual homozygote but obviously heterozygous for the silent gene. The paternal grandmother was deceased and both the paternal grandfather (W) and paternal uncle were found to be usual homozygotes. The cholinesterase temperature activity curves of the indicated members of this family are shown in Figure 8, the assay figures in Table 4 and the cholinesterase phenotype relationship in Figure 9.

Case 6

The patient (X) underwent orthopaedic surgery in Bridge of Earn Hospital, Perthshire. The parents were somewhat disquieted by reports of untoward reactions which they felt might have been a cardiac arrest. Professor H.G. Morgan thought that the episode could have been a scoline apnoea and took blood samples from the girl (X),

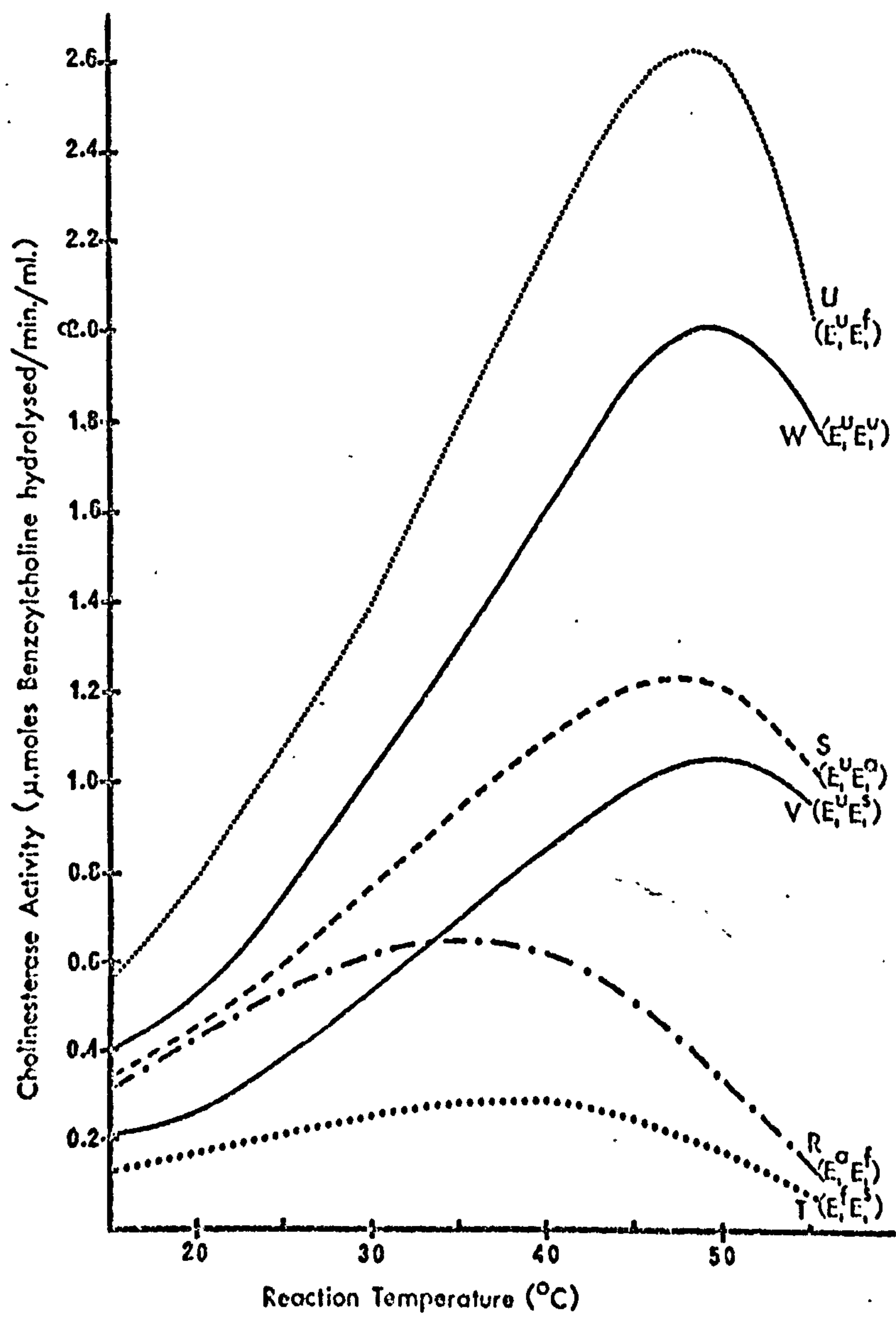


Figure 8. Cholinesterase temperature/activity curves of designated family members of Case 5 (page 52).

Table 4.

Assay Figures for the Family of Case 5

	Serum Cholinesterase (mU/ml at 25°C)	Dibucaine Number	Fluoride Number	Chloride Number	Phenotyp
Patient (R)	530	52	29	39	$E_1^a E_1^f$
Father (S)	585	60	48	23	$E_1^u E_1^a$
Mother (T)	210	62	33	28	$E_1^f E_1^s$
Maternal Grandfather (U)	1080	74	53	23	$E_1^u E_1^f$
Maternal Grandmother (V)	390	79	62	15	$E_1^u E_1^s$
Paternal Grandfather (W)	740	80	65	13	$E_1^u E_1^u$
Paternal Uncle	920	80	58	15	$E_1^u E_1^u$

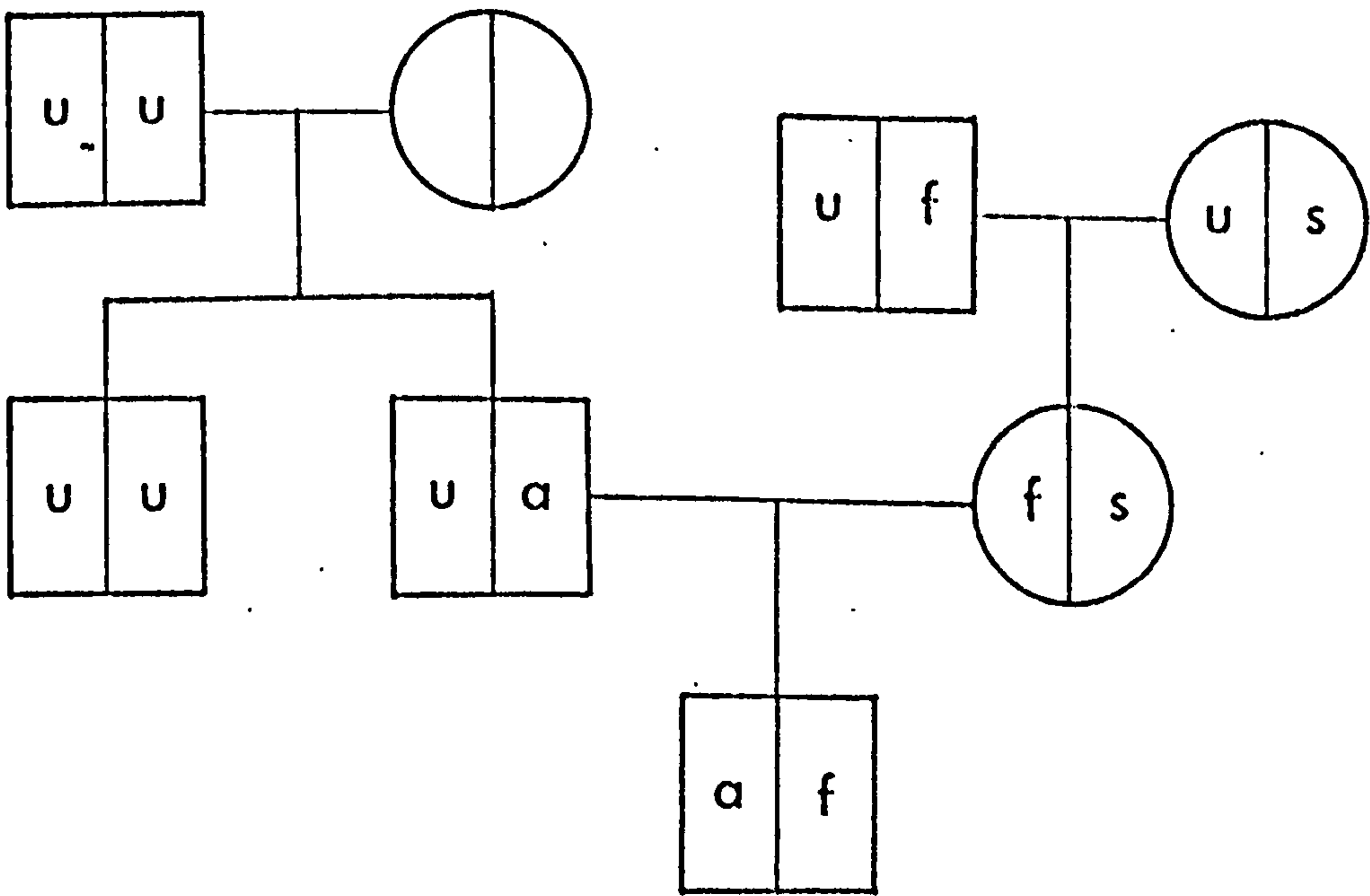


Figure 9. Phenotype relationship of Case 5 family (page 52).

her father (Y), mother (Z) and younger sister. Analysis of the sera indicated that the patient had cholinesterase activity less than 30 mU/ml and that the father, mother and sister were of the usual phenotype with serum activities of 385, 280 and 465 mU/ml at 25°C respectively. The patient was accordingly classified as homozygous for the silent gene, phenotype $E_1^S E_1^S$ and the parents as heterozygotes $E_1^u E_1^S$. The sister is probably also a usual/silent heterozygote.

The temperature activity curves for this family are shown in Figure 10.

Another family of which at least two were silent gene homozygotes was referred by Professor Richterich of Berne. However due to the untimely death of this renowned clinical biochemist most of the serum specimens and all the details of relationship and clinical data were not received.

From the curves of the foregoing cases which include all ten generally-acknowledged genotypes and from studies on other sera totalling 21 samples of $E_1^u E_1^u$, 6 of $E_1^u E_1^f$, 11 of $E_1^u E_1^a$, 3 of $E_1^f E_1^f$, 5 of $E_1^a E_1^f$ and 9 of $E_1^a E_1^a$ mean values were calculated relative to unit activity at 25°C, 30°C and 37°C. These temperature-activity relationships are illustrated in Figures 11, 12 and 13. Table 5 contains the Q_{10} values for all six phenotypes throughout the range of temperatures studied. Also derived from these studies, the temperature correction factors for the three homozygous phenotypes, $E_1^u E_1^u$, $E_1^f E_1^f$ and $E_1^a E_1^a$ are given in Tables 6, 7 and 8 respectively.

The cholinesterase activities at 25°C and 37°C of a

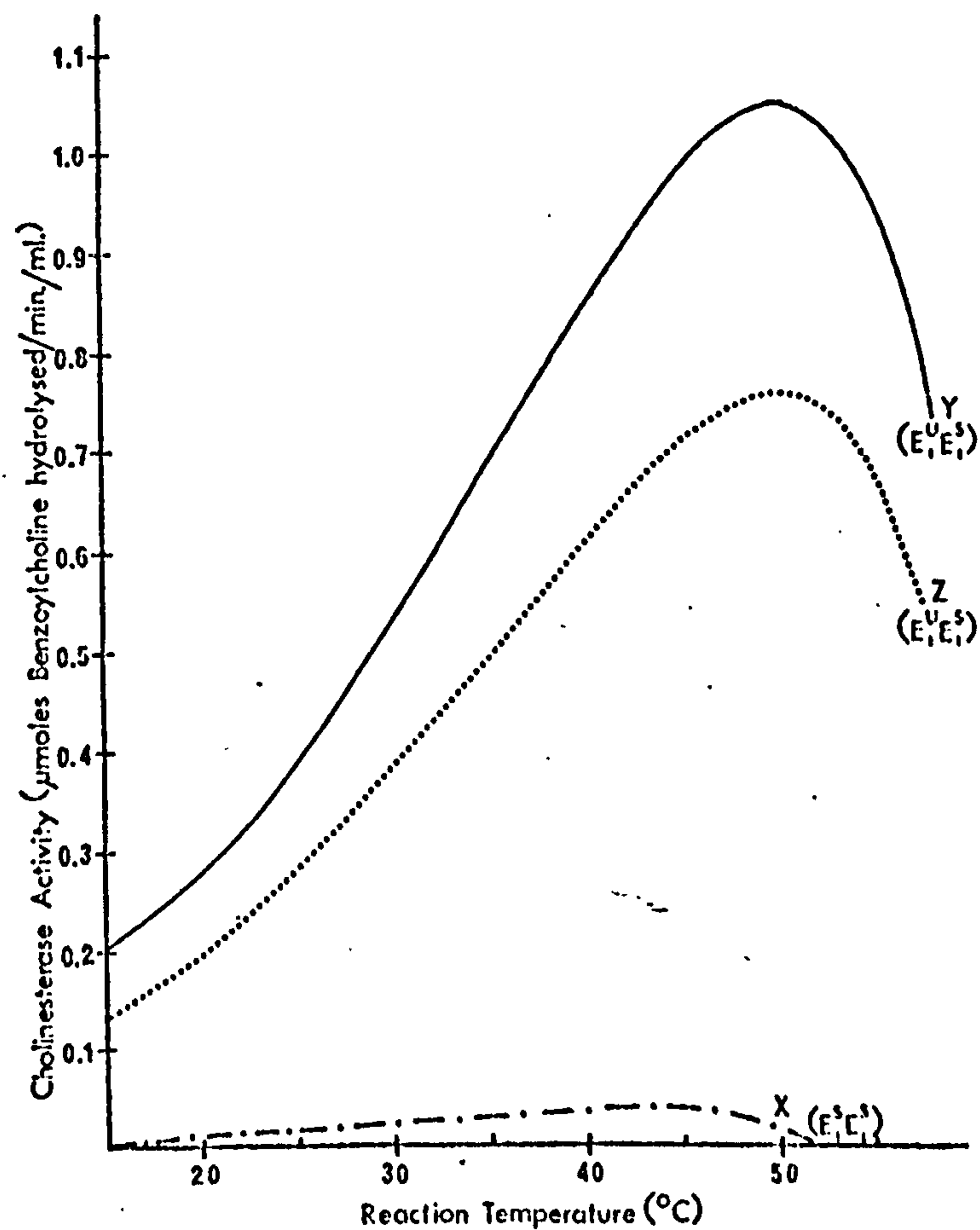


Figure 10. Cholinesterase temperature/activity curves of designated family members of Case 6 (pages 52, 53).

The rate of benzoylcholine hydrolysis by the propositus X was minimal and of the same order as the spontaneous hydrolysis. Despite this and the knowledge that at such levels of activity large proportional errors can result there was in several experiments a tendency for increase in the rate of hydrolysis with temperature exhibiting a maximum about 45°C.

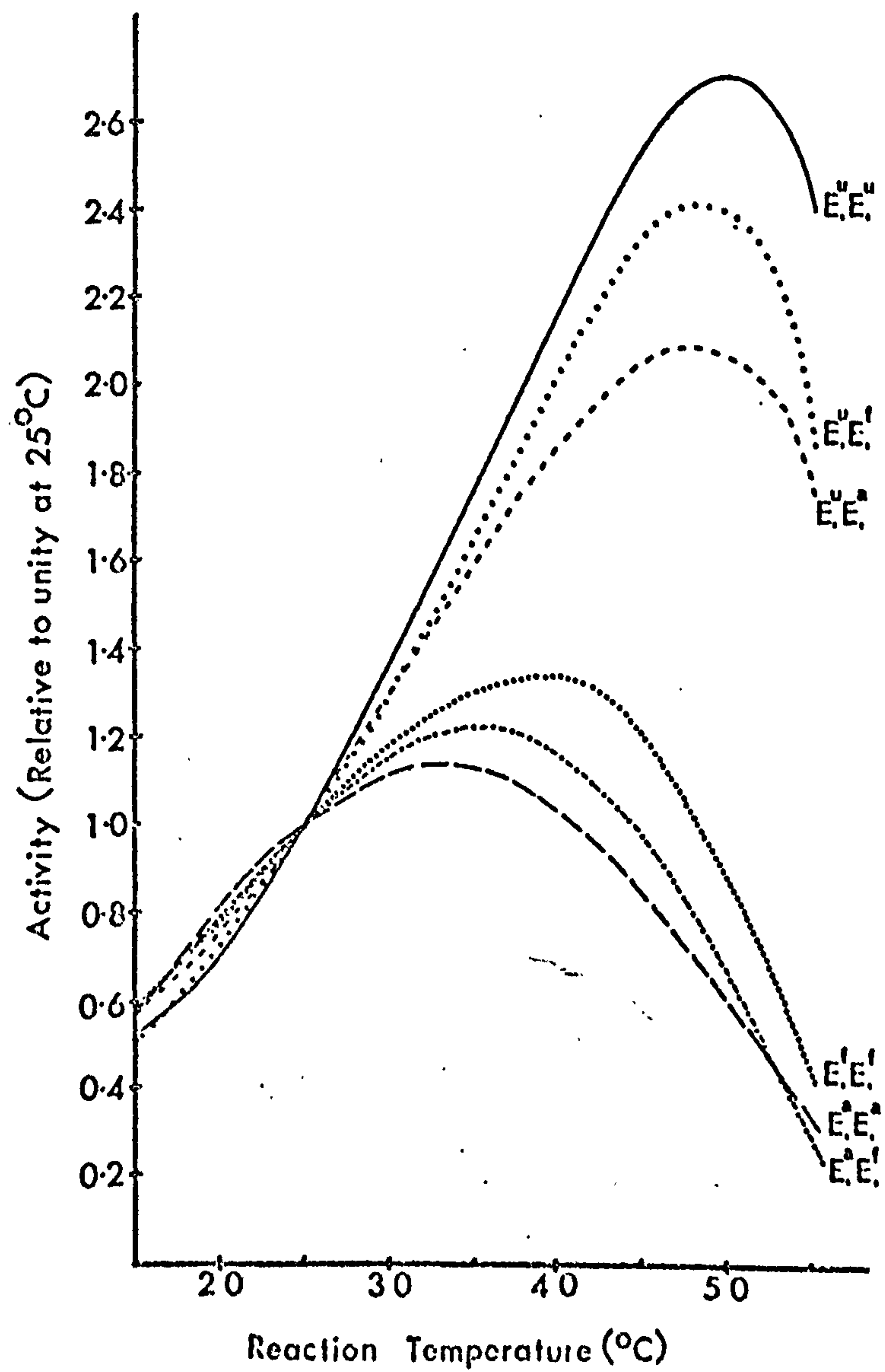


Figure 11. Temperature/activity relationships of serum cholinesterase phenotypes referred to unity at 25°C. Assay procedure of Kalow and Lindsay (1955).

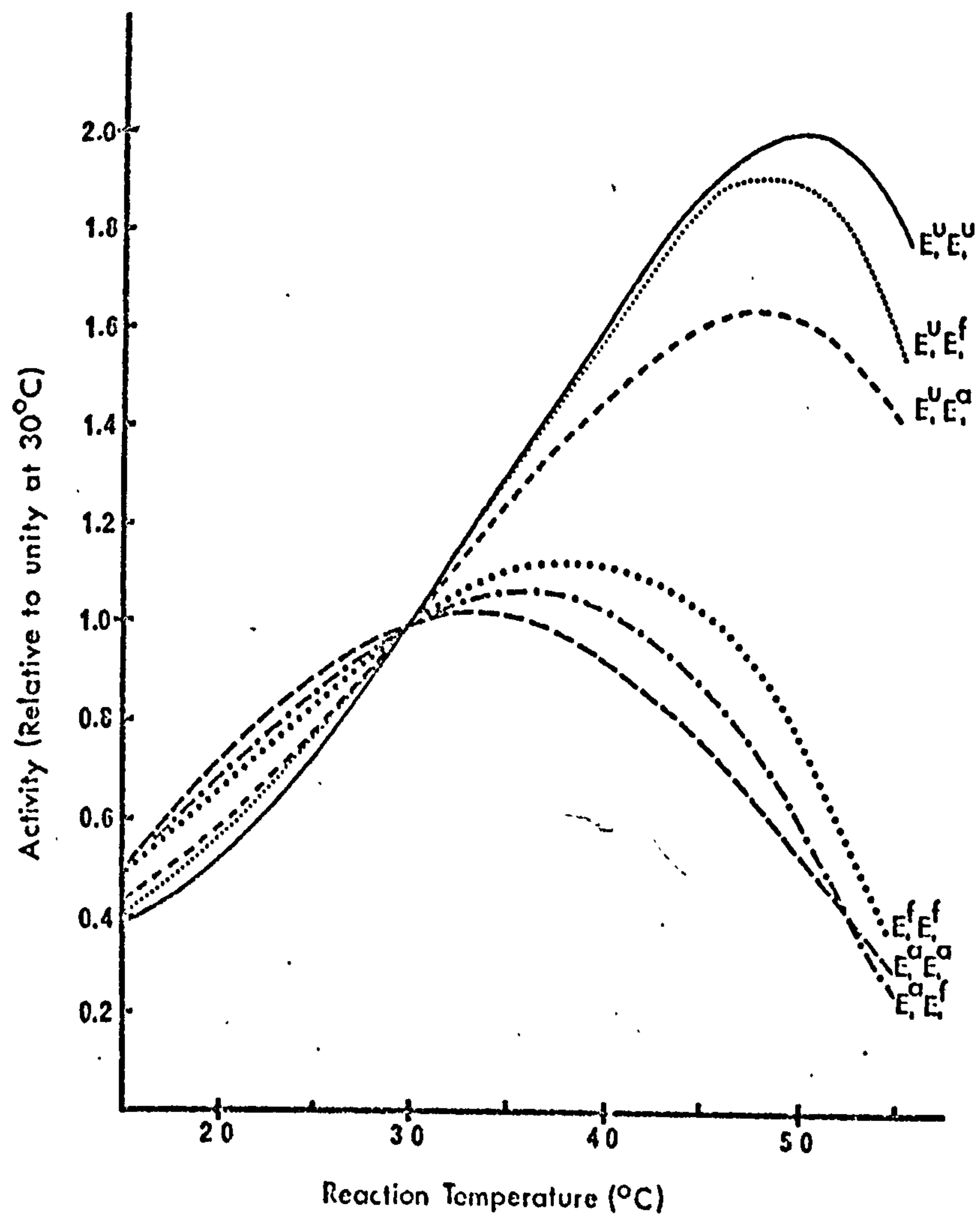


Figure 12. Temperature/activity relationships of serum cholinesterase phenotypes referred to unity at 30°C. Assay procedure of Kalow and Lindsay (1955).

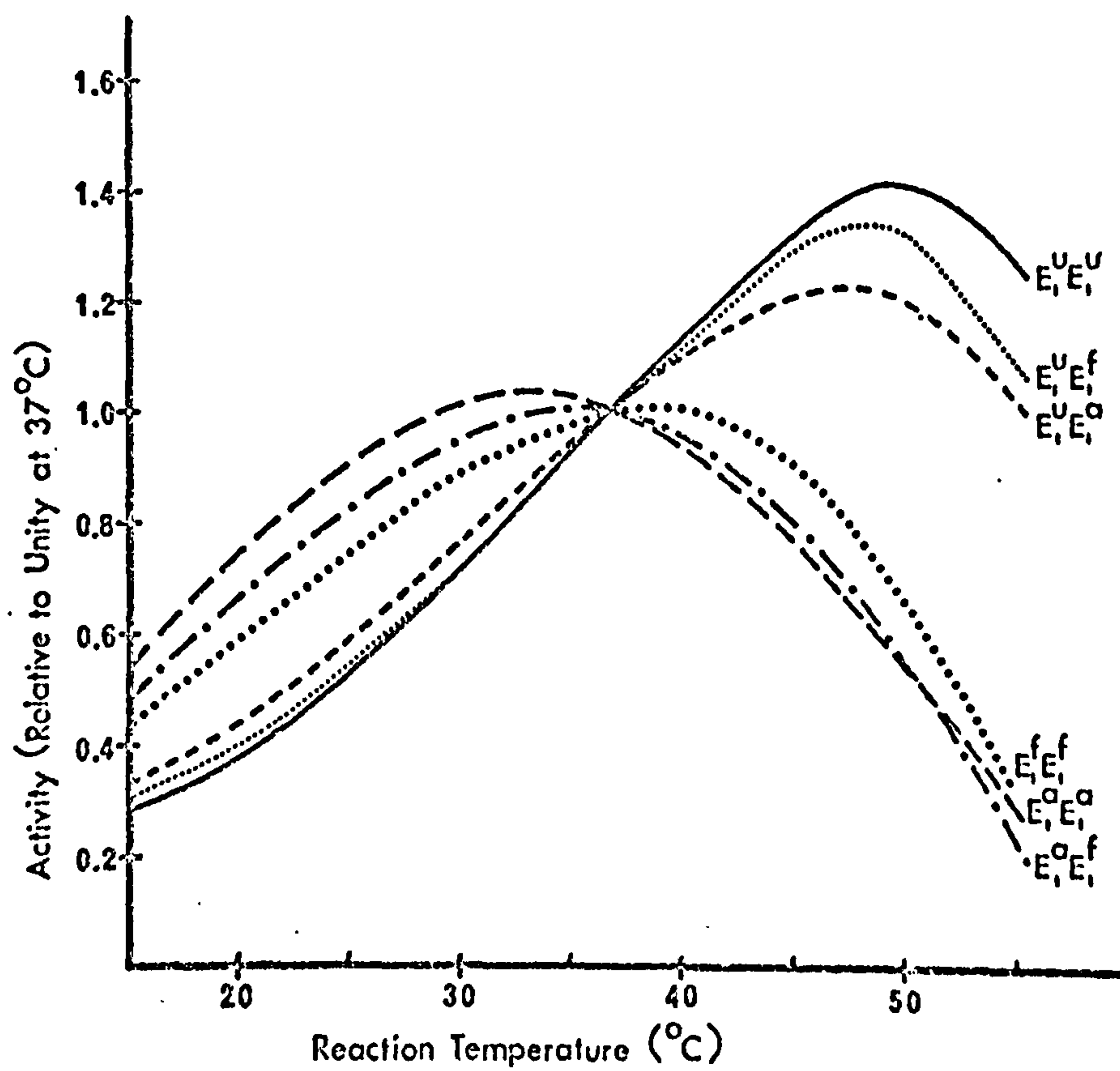


Figure 13. Temperature/activity relationships of serum cholinesterase phenotypes referred to unity at 37°C. Assay procedure of Kalow and Lindsay (1955).

Table 5.

Temperature Coefficients of Serum Cholinesterase Variants

Q_{10}	$E_1^u E_1^u$	$E_1^u E_1^f$	$E_1^u E_1^a$	$E_1^f E_1^f$	$E_1^a E_1^f$	$E_1^a E_1^a$
$15^{\circ} - 25^{\circ}\text{C}$	1.89	1.96	1.76	1.70	1.70	1.72
$20^{\circ} - 30^{\circ}\text{C}$	1.94	1.77	1.71	1.50	1.45	1.37
$25^{\circ} - 35^{\circ}\text{C}$	1.76	1.67	1.60	1.30	1.23	1.14
$30^{\circ} - 40^{\circ}\text{C}$	1.60	1.58	1.45	1.14	1.01	0.93
$35^{\circ} - 45^{\circ}\text{C}$	1.47	1.42	1.29	0.93	0.80	0.75
$40^{\circ} - 50^{\circ}\text{C}$	1.26	1.19	1.11	0.65	0.58	0.59
$45^{\circ} - 55^{\circ}\text{C}$	0.97	0.80	0.85	0.35	0.30	0.39

Table 6.

Temperature correction factors for serum cholinesterase phenotype E₁^u E₁^u

Reaction Temperature (°C)	Standard Temperature		
	25°C	30°C	37°C
15	1.89	2.58	3.62
16	1.80	2.45	3.44
17	1.70	2.32	3.25
18	1.61	2.19	3.08
19	1.52	2.08	2.92
20	1.42	1.94	2.72
21	1.33	1.81	2.54
22	1.24	1.69	2.37
23	1.15	1.57	2.21
24	1.07	1.47	2.06
25	1.00	1.37	1.92
26	0.94	1.28	1.80
27	0.88	1.20	1.68
28	0.82	1.13	1.58
29	0.78	1.06	1.49
30	0.73	1.00	1.41
31	0.69	0.95	1.33
32	0.66	0.90	1.26
33	0.63	0.86	1.20
34	0.60	0.82	1.14
35	0.57	0.78	1.09
36	0.54	0.74	1.04
37	0.52	0.71	1.00
38	0.50	0.68	0.96
39	0.48	0.65	0.92
40	0.46	0.63	0.88

Table 7.

Temperature correction factors for serum cholinesterase phenotype E₁^f E₁^f

Reaction Temperature (°C)	Standard Temperature		
	25°C	30°C	37°C
15	1.70	2.00	2.26
16	1.60	1.89	2.13
17	1.50	1.77	2.00
18	1.42	1.67	1.89
19	1.35	1.59	1.80
20	1.28	1.51	1.71
21	1.22	1.43	1.62
22	1.16	1.36	1.54
23	1.10	1.30	1.46
24	1.05	1.24	1.39
25	1.00	1.18	1.33
26	0.96	1.14	1.28
27	0.93	1.10	1.24
28	0.90	1.06	1.20
29	0.87	1.03	1.16
30	0.85	1.00	1.13
31	0.83	0.97	1.10
32	0.81	0.95	1.08
33	0.79	0.93	1.06
34	0.78	0.92	1.04
35	0.77	0.91	1.02
36	0.76	0.90	1.01
37	0.75	0.89	1.00
38	0.75	0.89	1.00
39	0.75	0.88	1.00
40	0.75	0.88	1.00

Table 8.

Temperature correction factors for serum cholinesterase phenotype E₁^a E₁^a

Reaction Temperature (°C)	Standard Temperature		
	25°C	30°C	37°C
15	1.75	1.96	1.95
16	1.61	1.80	1.80
17	1.50	1.67	1.67
18	1.39	1.56	1.55
19	1.30	1.46	1.45
20	1.23	1.38	1.37
21	1.17	1.31	1.30
22	1.12	1.25	1.24
23	1.07	1.20	1.19
24	1.03	1.16	1.15
25	1.00	1.12	1.11
26	0.97	1.09	1.08
27	0.95	1.06	1.05
28	0.93	1.03	1.03
29	0.91	1.01	1.01
30	0.89	1.00	1.00
31	0.88	0.99	0.98
32	0.87	0.98	0.97
33	0.87	0.97	0.97
34	0.87	0.98	0.97
35	0.88	0.98	0.98
36	0.89	0.99	0.99
37	0.90	1.00	1.00
38	0.91	1.02	1.02
39	0.93	1.05	1.04
40	0.96	1.08	1.07

wider selection of sera comprising 178 usual homozygotes, 24 usual/fluoride-resistant heterozygotes, 60 usual/atypical heterozygotes, 3 fluoride-resistant homozygotes, 11 atypical/fluoride-resistant heterozygotes and 20 atypical homozygotes are shown in Figures 14 and 15.

Discussion

Not all prolonged responses to suxamethonium can be attributed to enzymic anomalies and indeed may not be attributable to suxamethonium alone but may be caused by a complex situation dependent upon anaesthetic premedication or simple reduction in cholinesterase activity in conjunction with electrolyte imbalance (Kalow, 1959a). That there exist routes of inactivation or removal of suxamethonium apart from hydrolysis by serum cholinesterase is shown by the recovery from suxamethonium apnoea, albeit slow, of individuals homozygous for the silent gene and therefore devoid of the enzyme (Hart and Mitchell, 1962; Liddell, Lehmann and Silk, 1962). It may well be a failure of these supporting or alternative mechanisms which results in prolonged paralysis in individuals for whom no genetic anomaly can be demonstrated (Telfer, McDonald and Dinwoodie, 1964).

However, a low serum cholinesterase level alone is rarely a cause for concern in the use of suxamethonium while the mass of evidence accumulated over the last decade or so concerning the inherited variants of serum cholinesterase has indicated that individuals homozygous for one of the variant species ($E_1^S E_1^S$, $E_1^a E_1^a$, $E_1^f E_1^f$) or heterozygous for two ($E_1^S E_1^a$, $E_1^S E_1^f$, $E_1^a E_1^f$) show undue sensitivity to suxamethonium. Individuals homozygous or heterozygous for the usual enzyme ($E_1^u E_1^u$ or $E_1^u E_1^S$, $E_1^u E_1^f$, $E_1^u E_1^a$) do not usually exhibit prolonged response (Kalow, 1959; Lehmann, Liddell, Blackwell, O'Connor and Daws, 1963). The rate of occurrence of suxamethonium apnoea among the different

phenotypes has been summarised by Lehmann and Liddell (1969).

Kalow (1959) demonstrated that compared to the usual enzyme, the atypical or dibucaine-resistant enzyme had a reduced affinity for succinylcholine and calculated that at in vivo concentrations the drug would not combine with the atypical esterase. This last postulate took no account of the relatively increased concentration, at least one-hundred fold, of enzyme in vivo compared to the in vitro experiments but certainly a reduced efficiency in hydrolysing suxamethonium was demonstrated for the atypical enzyme. Where apnoea is connected with an inherited cholinesterase variant it has therefore been assumed that the sole cause is the reduced affinity for suxamethonium and this was summed up by Wylie and Churchill-Davidson (1966) as follows: "The only difference between these two enzymes is one of degree. Both are capable of hydrolysing suxamethonium in vitro but only the normal enzyme can do it in clinical conditions where the concentration of suxamethonium is low. Thus if a patient with the atypical enzyme receives a dose of suxamethonium, the dilution caused by the blood volume rapidly causes a drop in its concentration below the effective level for the atypical esterase. In these circumstances the patient remains paralysed for a long period of time".

The results of the present study indicate however that the reduced enzyme-suxamethonium affinity is not the sole factor causing the increased time of paralysis through decreased hydrolysis of the drug. There is also the

inactivation at body temperature of the enzyme variants present in the serum of scoline-sensitive subjects which reduces the rate of hydrolysis. At least this has been conclusively shown in the foregoing studies with benzoylcholine and was so reported (King and Dixon, 1969). It was realised that this might not be the case with other substrates, in particular with succinylcholine and therefore this was examined and is discussed later, (page 103 et seq.)

These studies also show that the presence of a silent gene does not affect the temperature-activity characteristics of the allele present in heterozygotes. In this respect the characteristics of the usual/silent heterozygote $E_1^u E_1^s$ shown in loci F and H (Figure 4) and V (Figure 8) are identical with the usual homozygote curves, C (Figure 3), N (Figure 6) and W (Figure 8). Similarly the curve of the atypical/silent heterozygote $E_1^a E_1^s$ of G (Figure 4) is identical with curve A (Figure 3) given by an atypical homozygote. Curve T (Figure 8) that of genotype $E_1^f E_1^s$ has the same temperature-activity characteristics as those of M and Q (Figure 6) given by the fluoride-resistant homozygotes. This was to be expected, has already been noted in inhibition studies and was confirmed by the investigation of the only silent homozygote to be discovered in this study; curve X (Figure 10). This does not deny that there may in some cases be minor but probably undetectable variations due to the silent variant since this has been shown conclusively to be a heterogeneous group (Goedde and Altland, 1968; Rubinstein, Dietz, Hodges, Lubrano and Czebator, 1970).

Harris, Lehman and Silk (1960) reported that there was "a significant correlation between the dibucaine numbers and cholinesterase levels within the 'intermediate' phenotype". This has been generally confirmed in the present study with the outstanding exception of individual D of Case 1. However, the cholinesterase activity of cord blood serum is generally lower than adult normal (Lehmann, Cook and Ryan, 1957; King, 1965) and this probably accounts for the low activity found in that case (Figure 3). Dibucaine and fluoride numbers were readily determined on this specimen which also yielded a curve similar to those found in the adult phenotype. It is perhaps noteworthy that at 25°C at which activity was measured this neonate's serum cholinesterase activity was only a little higher than that of the mother A (Figure 2) while at 37°C, the functional temperature of the enzyme, the cord serum activity was nearly twice as high and this alone indicates some reduction of risk in the use of suxamethonium.

This single example of the general findings reported above also helps to resolve some anomalies noted in the literature. Bush (1961) for instance found "a wider scatter of cholinesterase levels in the heterozygote and abnormal homozygote groups" than that observed by Lehmann, Patston and Ryan (1958) and later by Harris, Lehmann and Silk (1960). All these workers used acetylcholine as substrate, but Bush (1961) employed the procedure of Biggs, Carey and Morrison (1958) in which the pH change is measured during 30 minutes at 25°C, while Lehmann and colleagues recorded the volume of carbon dioxide liberated in a

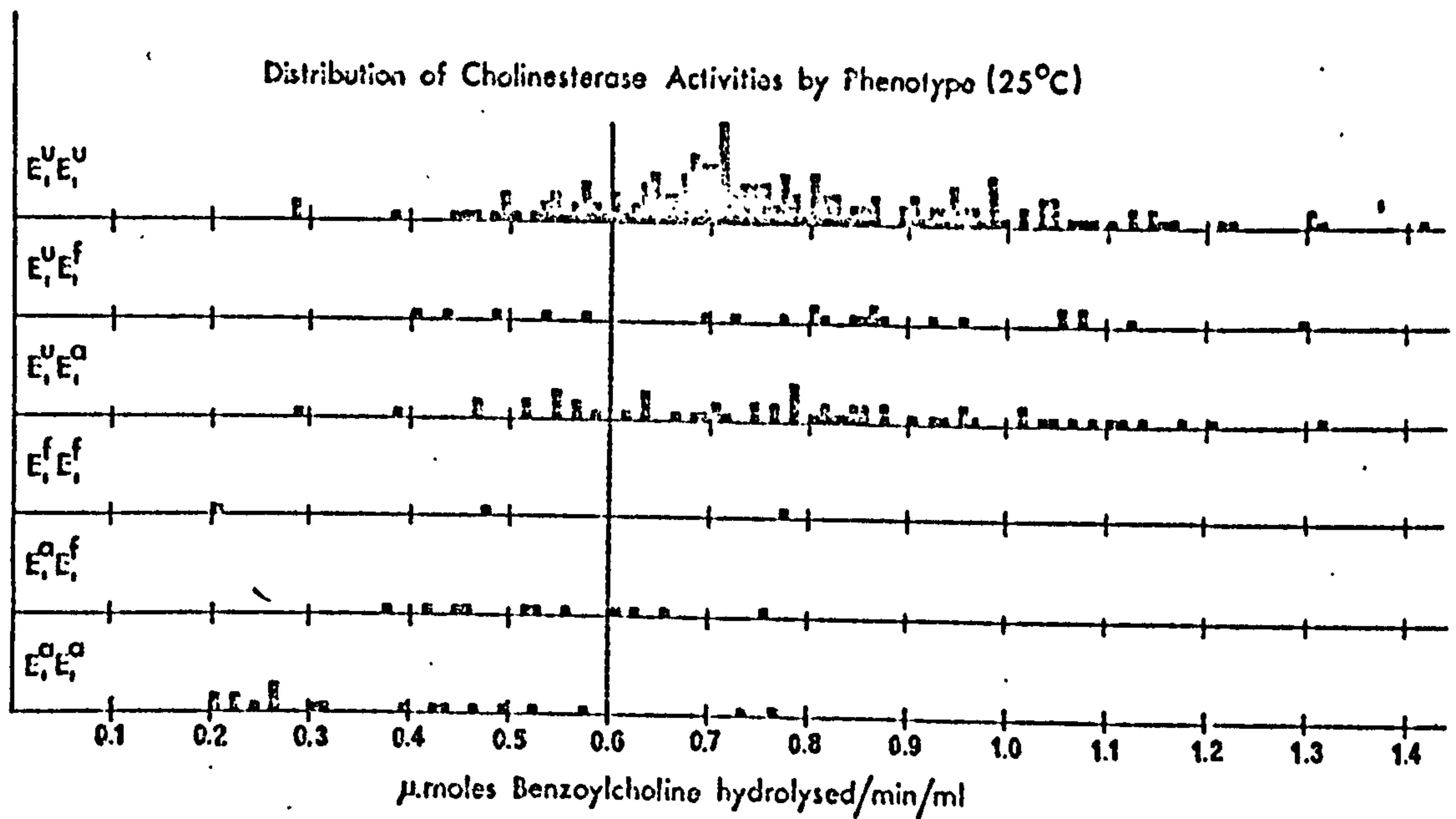


Figure 14. Serum cholinesterase activities by phenotype assayed at 25°C by the procedure of Kalow and Lindsay (1959). The vertical broken line indicates the lower limit of normal activity (King, 1965).

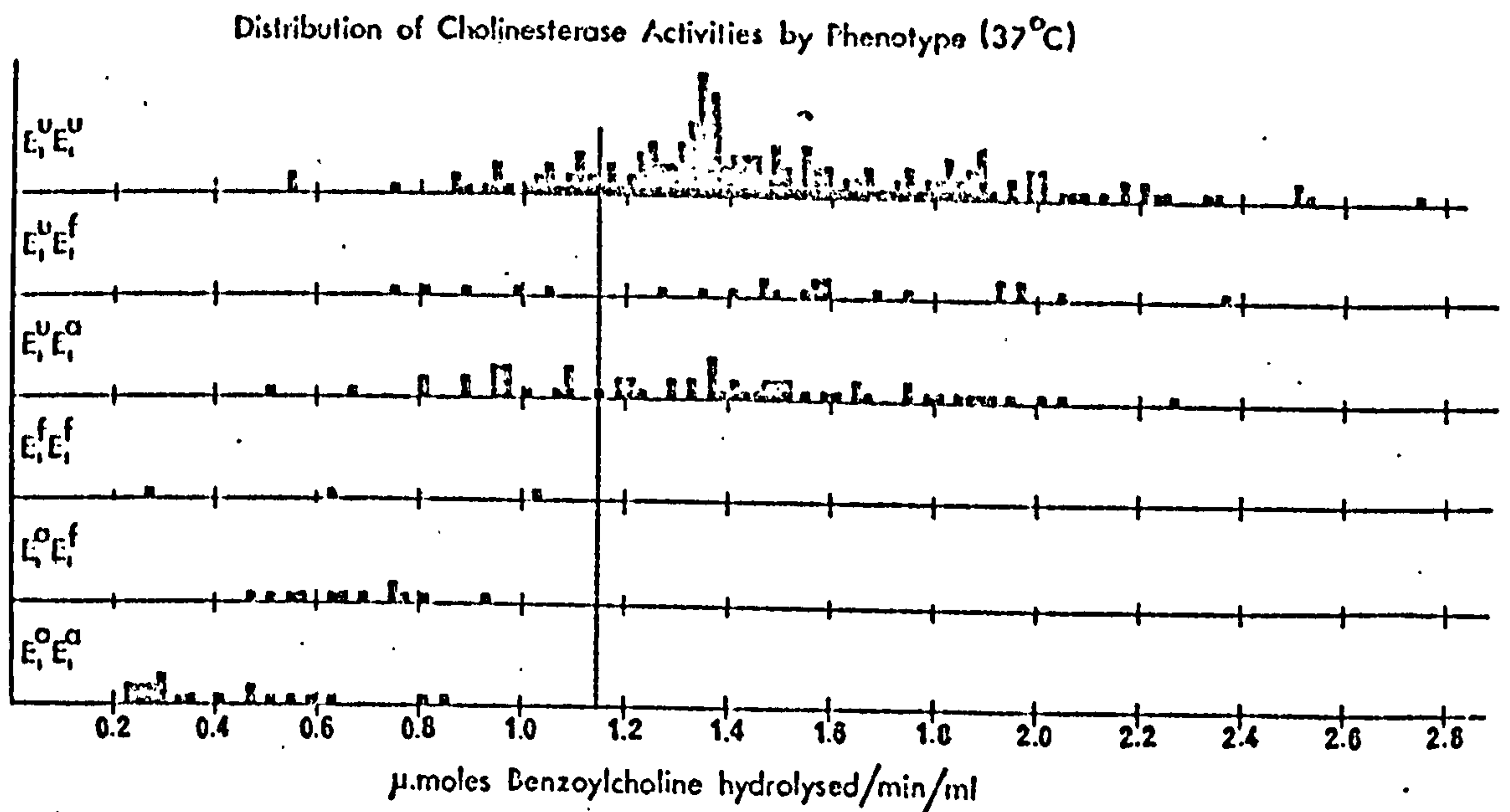


Figure 15. Serum cholinesterase activities by phenotype assayed at 37°C by the procedure of Kalow and Lindsay (1955). The vertical broken line indicates the lower limit of normal activity (King, 1965).

Warburg micromanometer at 37°C. From the results of these present studies it is obvious why the latter workers obtained at 37°C a better separation of the phenotypes by assay alone and indeed emphasises the fact that a true index of serum cholinesterase activity can only be obtained if the estimation is conducted at the functioning temperature of the enzyme, that is, 37°C. This is reinforced by the values shown in Figures 14 and 15 of activities on the same specimens by assay at 25°C and 37°C respectively. The usual homozygote group illustrated in these figures is by no means representative of a normal population and contains at least six, presumptive but almost certain, usual/silent heterozygotes and a number of hospital patients, some suffering from diseases in which low cholinesterase levels are to be expected. Despite this it is readily seen that there is a wide overlap of activities from all phenotypes at 25°C (Figure 14) and that a number of suxamethonium-sensitive individuals have serum cholinesterase activities which can only be classed as normal. While with activities at 37°C (Figure 15) the distribution of the usual homozygotes does not alter significantly, a larger proportion of the usual/atypical heterozygote group have activities which fall below the lower limit of normal. Most conclusively however is the change in distribution of the three scoline-sensitive phenotypes where all activities are seen to be abnormally low. This is one of the facts used by King (1972) in recommending the adoption of 37°C as the standard temperature for enzyme assays.

Certainly if cholinesterase estimations are carried

out at any other temperature then for valid results these must be corrected to 37°C. This would involve determining the phenotype and then employing the appropriate set of temperature correction factors. King and Morgan (1970) suggested that for routine purposes this could be simplified without incurring a gross error by considering two groups only. In support of this it was noted that the phenotypes $E_1^u E_1^u$, $E_1^u E_1^f$ and $E_1^u E_1^a$ have respectively Q_{10} (25° - 35°C) of 1.76, 1.67 and 1.61 and correction factors from 25°C to 37°C of 1.92, 1.82 and 1.72. This represents a maximum divergence of about 11% in the range 25° - 37°C. Again the variants $E_1^a E_1^a$ and $E_1^a E_1^f$ have Q_{10} (25° - 35°C) of 1.14 and 1.23 and 25°C to 37°C conversion factors of 1.11 and 1.22 respectively, a divergence of less than 10%. Later (King and Morgan, 1971) it was noted that for the $E_1^f E_1^f$ phenotype the Q_{10} (25° - 35°C) was 1.30 and the correction factor from 25°C to 37°C was 1.33, a divergence of nearly 20% from the atypical homozygote values and more than 30% from the normal homozygote figures. This suggested a third set of temperature correction factors were required. Such figures to convert activities to 25°C, 30°C and 37°C the temperatures recommended (International Union of Biochemistry, 1961; 1965) or commonly used as standard temperatures, for the usual enzyme, the fluoride-resistant variant and the atypical cholinesterase are given in Tables 6, 7 and 8 respectively.

It is realised that these figures only apply to a benzoylcholine substrate and in fact only to a 50 µM benzoylcholine concentration under the assay conditions

used in these studies. These are relevant facts which dispute the validity of using temperature correction factors and reinforce the arguments for international adoption of a standard assay temperature in clinical enzymology (King, 1972).

From these studies the ratio of activities at 37°C and 26°C is 1.78 and the temperature coefficient (Q_{10}) in the range $15^{\circ}\text{C} - 25^{\circ}\text{C}$ is 1.90 for the usual enzyme. These figures are in excellent agreement with the values given by Kalow and Lindsay (1955). The literature does not contain comparable thermal characteristics for the other serum cholinesterase variants.

Reports that the magnitude and duration of suxamethonium paralysis are increased by hypothermia (Zaimis, Cannard and Price, 1958; Cannard and Zaimis, 1959) are in keeping with the facts presented here (Figure 12) although it is doubtful if this phenomenon can be attributed solely to the decrease in serum cholinesterase activity with reduction in body temperature. In fact this effect should be less in suxamethonium-sensitive individuals and indeed it is interesting to speculate that whatever the function of serum cholinesterase (Clitherow, Mitchard and Harper, 1963) those individuals of phenotypes $E_1^a E_1^a$, $E_1^a E_1^f$ and $E_1^f E_1^f$ will be less affected by a reduction in body temperature than will normal individuals.

Under the assay conditions employed in these studies, the temperatures at which maximal activities are obtained is $50 - 51^{\circ}\text{C}$ for the usual enzyme, about 48°C for the usual heterozygotes $E_1^u E_1^f$ and $E_1^u E_1^a$, 40°C for the fluoride-

resistant homozygote, about 36°C for genotype $E_1^a E_1^f$ and $32 - 33^{\circ}\text{C}$ for the atypical homozygote. The results of the denaturation experiments (Table 2) would therefore indicate that only in the case of the usual enzyme is the optimal temperature dictated by an irreversible process and that the temperature of maximal activity for the other variants is set by a progressive, reversible inactivation. This constitutes a previously undescribed phenomenon of isoenzyme differentiation and indeed would appear to contradict Harris (1970), who, referring to the usual and atypical enzymes, stated "However it should be noted that in a number of properties, for example thermostability and electrophoretic mobility the two forms of the enzyme do not apparently differ". This contradiction results from the earlier approaches to thermal differentiation which had relied, as in Table 2 experiments, upon the irreversible effect of heat on the protein itself rather than on its catalytic activity.

It is possible, if not to differentiate each phenotype, at least to segregate, suxamethonium-sensitive individuals from those at no predictable risk, by means of assays at two temperatures. The two most feasible temperatures in the light of present discussions on standard temperature would appear to be 25°C and 37°C or 30°C and 37°C . This would provide a method of differentiating the usual homozygote and heterozygotes from the $E_1^a E_1^a$ and $E_1^a E_1^f$ variants, that is, those most at risk to succinylcholine. The latter group have $37^{\circ}/25^{\circ}$ and $37^{\circ}/30^{\circ}$ ratios of less than 1.3 and less than 1.1 respectively while for the

former group these temperature ratios are 1.7 to 2.0 and 1.3 to 1.5. It is not possible to differentiate further within these two groups with certainty and King and Morgan (1970) further predicted that the phenotype $E_1^f E_1^f$ would be found to occupy a position intermediate to the two groups. In fact the fluoride-resistant homozygote was found to be closer to the other suxamethonium-sensitive phenotypes than expected with $37^\circ/25^\circ$ and $37^\circ/30^\circ$ activity ratios of about 1.35 and 1.15 (King and Morgan, 1971). This makes segregation less certain using 30°C and 37°C as the assay temperatures but the difference between the sensitive and non-predictable groups is adequate when 25°C and 37°C are the temperatures employed. Differentiation would obviously be improved with greater divergence in the two temperatures. One hesitates to employ 50°C which is close to the temperature of denaturation but assays at 25°C and 45°C would give ratios of activities about 0.85 and 1.0 for the phenotypes $E_1^a E_1^a$ and $E_1^a E_1^f$ respectively, about 1.2 for the fluoride-resistant homozygote and from 2.0 through 2.35 to 2.65 for phenotypes $E_1^u E_1^a$, $E_1^u E_1^f$ and $E_1^u E_1^u$. This could readily form the basis for a simple screening test of surgical patients for suxamethonium-sensitivity.

However at this time there was no means of recording reaction rates at 240 nm with such temperature differentials which could be mechanised to give through-put rates compatible with screening procedures. The Unicam AC 60/1800 system was the only available instrumentation which seemed to have the capability. In co-operation with Mrs. Christine Witchell of the Applications Department of Pye-Unicam,

Cambridge a study involving the use of inhibitors (Witchell, 1971) and of different assay temperatures was carried out. The temperature change proved cumbersome and time-consuming and even reducing the scan-time to two minutes only permitted processing ten to fifteen specimens per hour. In addition, the instrumentation embodied no automatic range selection and permanent attendance at the spectrophotometer was essential. In later models of this spectrophotometer, Unicam have improved the sensitivity and introduced automatic adjustment of the absorption range so that screening for suxamethonium-sensitivity may now be a feasible proposition with this instrument.

Continuous-flow mechanisation, as in the Auto-Analyser systems is not capable of yielding valid enzyme reaction rate assays nor apparently even valid two-point determinations. In enzyme estimations the time of the reaction must be precisely known and the reaction temperature must be constant throughout the reaction. While it would seem possible to arrange these conditions, the writer is not aware of any fixed-time enzyme assay by continuous flow technology which does. Again the simple filter colorimeters employed in Auto-Analyser systems do not permit of recording absorptions at 240 nm. However, if a ratio of activities, as opposed to an absolute activity is all that is required then assays by continuous-flow mechanisation are permissible. The hydrolysis of thiocholine esters may be followed at 405 nm using Ellman's reagent and this removes the problem of recording absorptions in the ultra-violet range with a benzoylcholine substrate. This seemed an

eminently practical procedure and Dr. E.D. Lash of Technicon Instruments Corporation, Tarrytown, New York expressed interest and was given the temperature-activity data and different phenotype samples. By an ingenious arrangement of splitting the sample-reactant stream into water baths at 37°C and 55°C and by arranging for the signals from both colorimeters to be simultaneously drawn on the same recorder with peak heights made equal with usual homozygote sera, the presence of a suxamethonium-sensitive phenotype could be detected by a simple scrutiny of the chart (Lash, Ponzio, Rush and Garry, 1972).

Further experiments and studies in the field of mass screening are more fully and appropriately described later (page 103 et seq.).

As recorded earlier (page 10 et seq.) variations in cholinesterase activity occur in certain disease states and in pregnancy (page 123) and some such specimens are included in Figures 14 and 15. Apart from this even in a normal healthy population the scatter of serum cholinesterase activities is regarded as wide and contradictory reports and speculations abound to account for this.

There would certainly appear to be a positive correlation between body weight and cholinesterase activity (Thompson and Trouce, 1956; Kalow and Gunn, 1959) and this factor may in turn be the major influence in the higher activities reported in males (Reinhold, Tourigny and Yonan, 1953; Breuer and Schönfelder, 1961) or with age (Kalow and Gunn, 1959). In infancy, after the first week of life (Lehmann, Cook and Ryan, 1957) and in children

(King, 1965) significantly higher activities are reported. These influences have either not been observed by other workers or positively refuted (Wetstone, Honeyman and McComb, 1965).

These last workers from a study of twins, parents and sibs concluded on statistical evidence that there was a major genetic control of quantitative levels of cholinesterase activity. On the other hand, Simpson and Kalow (1963) from a similar examination of monozygotic twins, like- and unlike-sexed dizygotic twins, sibs and parents concluded that the observed variation in cholinesterase activities was largely environmentally determined and found no evidence for any important homogenic or multigenic control.

With regard to the wide range of cholinesterase activity and its quantitative control it has not been clearly stated that this may be due to two possible causes. The first is an alteration in the rate of synthesis and could be caused either by a mutation in, or other influence on, a regulator gene, or by a similar effect in an unrelated gene resulting in a change of some inducer or repressor substance. This would involve a change in the actual number of enzyme molecules produced. The second cause could be the synthesis of an altered enzyme protein with consequent changes in either catalytic efficiency or structural stability. This does not envisage a change in the number of enzyme molecules produced, but a quantitative change in activity or in vivo stability secondary to a qualitative change in the enzyme.

The reduced catalytic activity of the unusual variants is readily appreciated (Figure 15) but of these only the genotypes $E_1^u E_1^f$ and $E_1^u E_1^a$ occur in a significant proportion of the population, 0.4% and 4% respectively (Lehmann and Liddell, 1969). These therefore contribute to the observed scatter of serum cholinesterase activities. However this was appreciated by the protagonists of the genetic control of quantitative enzyme activity who accordingly selected their cases from the "usual" phenotype. Although they did not specify the mode of control it is therefore evident that they meant factors influencing the rate of synthesis as outlined above. About 95% of the population are phenotype $E_1^u E_1^u$ and about 0.5% are probably genotype $E_1^u E_1^s$. The latter certainly have reduced cholinesterase activity. The C_5 variant E_2^+ , which confers additional cholinesterase activity occurs in about 10% of the population (Harris, Hopkinson and Robson, 1962). Already therefore in a selected phenotype, that regarded as normal, there are two inherited factors contributing to an extension at both ends of the "normal" range of activities. If these influences are eliminated from the population to be investigated it can be reasoned that the range of serum cholinesterase activities will be much reduced. Only then would the population also be free from the second cause of genetic quantitative control previously outlined.

As it is the range of serum cholinesterase activities is not wide compared to other enzymes; the upper limit of normal is only twice the lower limit. For the normal ranges of aspartate transaminase and alanine transaminase

activities in serum the upper limits are four to five times the lower limits (King, 1973). The normal serum range for creatine kinase activities is 0 to 100 mU/ml, and this is doubtless environmentally and to a lesser extent hormonally controlled. The higher numerical values attached to serum cholinesterase activities are merely a reflection of its "serum specificity" (page 11).

2. (b) Arrhenius Relationships.

Preliminary studies had indicated that the 50 μM benzoyl-choline employed in these studies although optimal for the usual enzyme at 25°C was grossly suboptimal for the other allelic isoenzymes. At 25°C the atypical enzyme exhibited maximal velocity with 100 μM benzoylcholine and this was the concentration required for substrate saturation of the usual enzyme at 37°C. It was realised that the activities recorded in the previous experiments did not, for the most part, represent maximum velocities and that the differences in thermal inactivation might be due to differences in substrate concentration requirements rather than true reversible alterations in protein structure. Specimens of the three homozygous sera were therefore assayed through the temperature range at both 50 μM and 100 μM benzoylcholine concentrations. The results shown in Figure 16 would appear to indicate that the temperature of maximum activity, or more precisely, the temperature of zero activation energy is a reflection of the protein structure and relatively independent of the substrate concentration, although some substrate protection may be afforded at the temperature of irreversible denaturation.

Arrhenius plots (Figure 17) taken from the data of Figure 11 reveal that the customary linear relationship can only be said to exist for the usual homozygote and heterozygotes and that over a very limited temperature range below 30°C. While Figure 18 would appear to indicate that linearity of the relationship is extended by the use of 100 μM benzoylcholine this is probably fallacious since at this concentration substrate inhibition is encountered

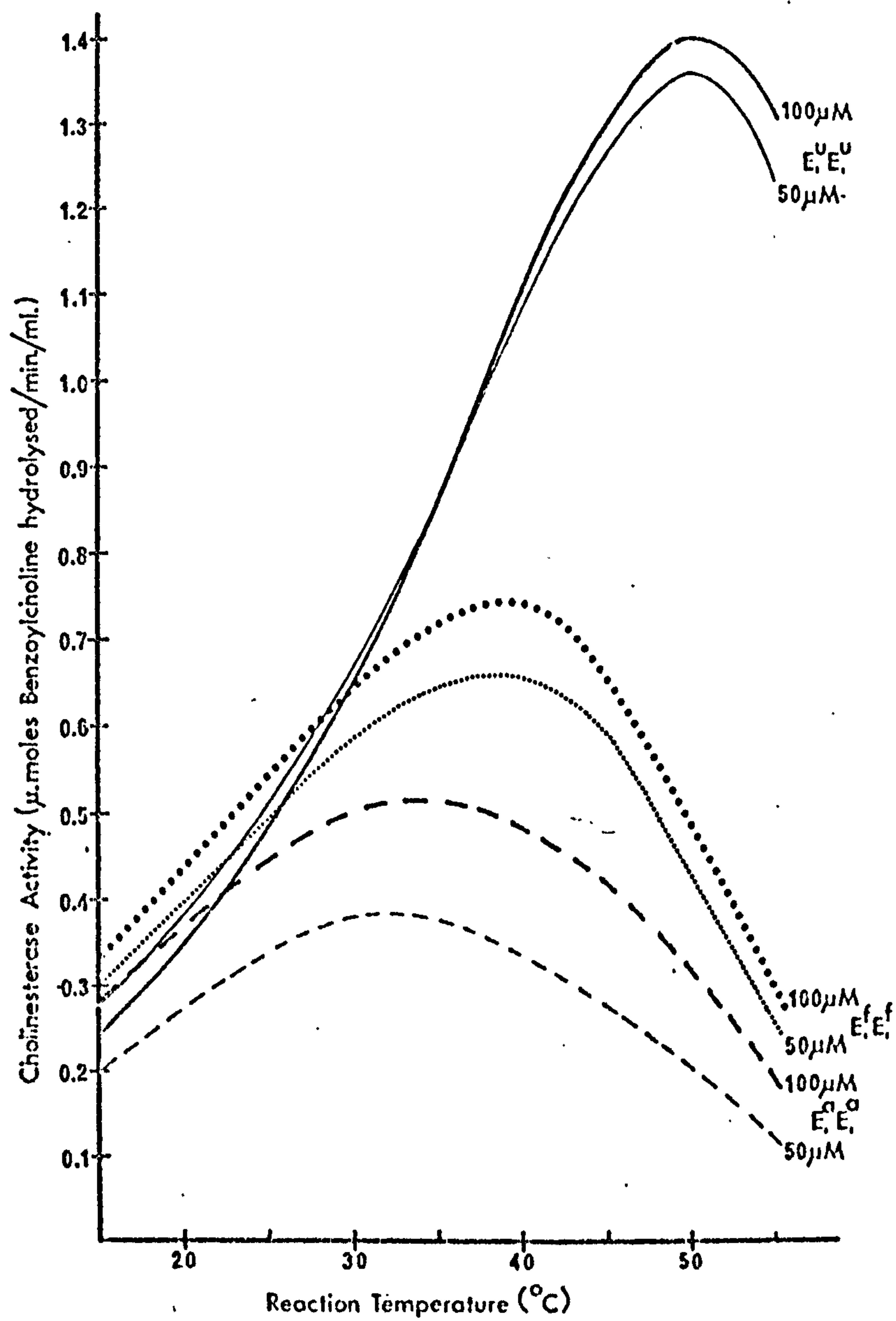


Figure 16. Temperature/activity relationships of the three cholinesterase homozygotes with 50 μM and 100 μM benzoylcholine substrate in phosphate buffer, pH 7.4.

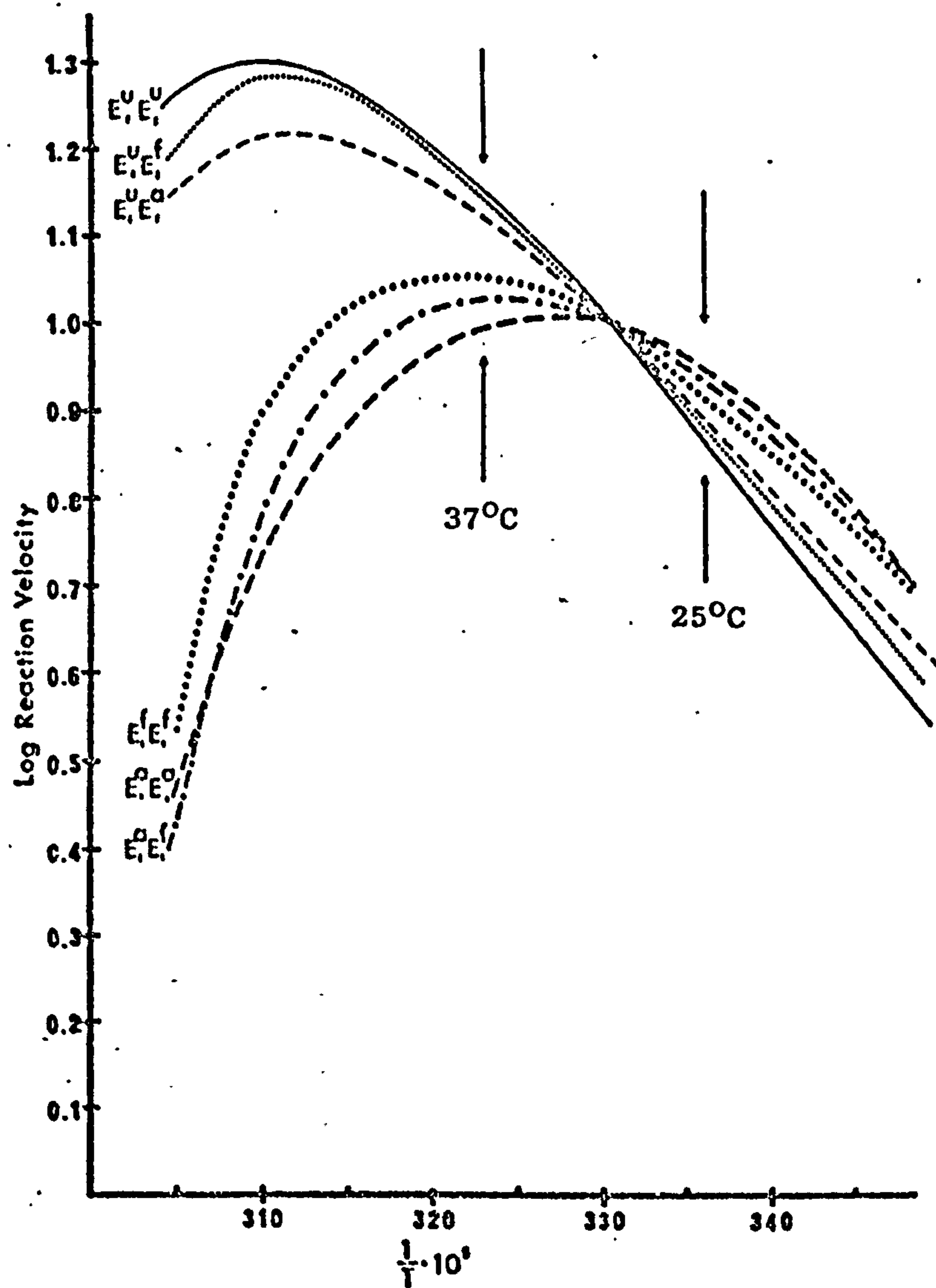


Figure 17. Arrhenius relationships of serum cholinesterase phenotypes. These plots are taken from data of Figure 12 in which activities were referred to unity at 30°C.

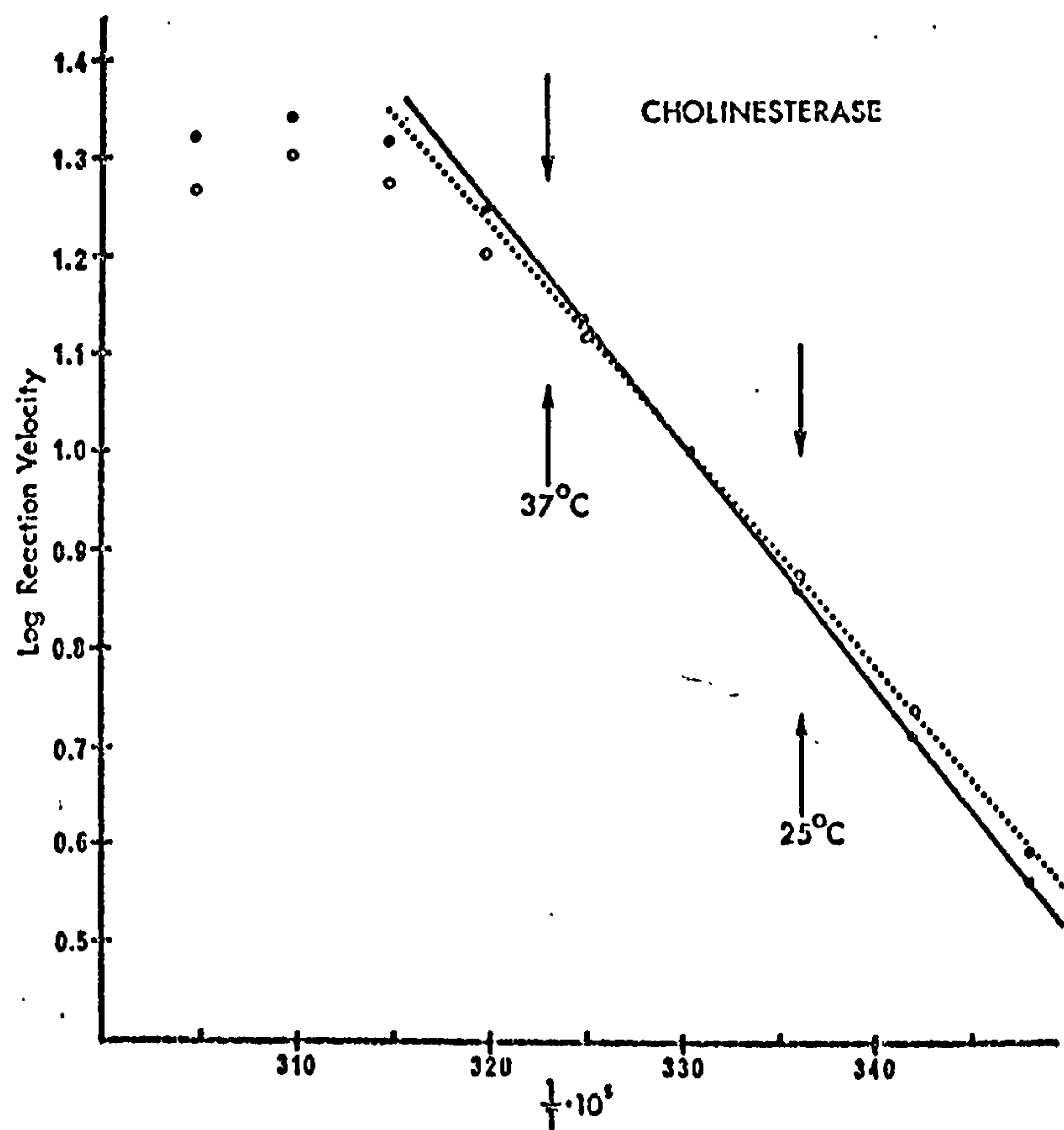


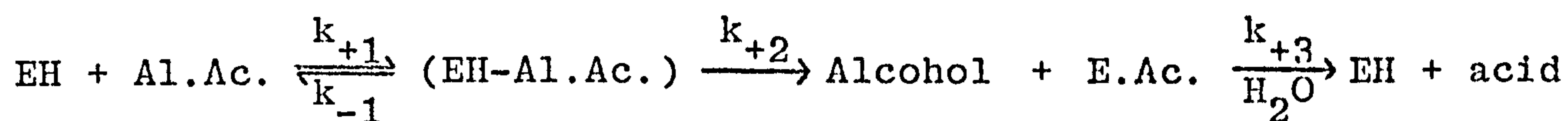
Figure 18. Arrhenius relationships of the usual cholinesterase enzyme at benzoylcholine concentrations of 50 μ M and 100 μ M.

at temperatures below 30°C (Figure 16) and this doubtless contributes to the extended linearity of the locus. This extended linearity with 100 µM benzoylcholine is therefore spurious and emphasises that a valid temperature-activity relationship can only be established by use of reaction conditions which are optimal at each temperature.

As it is these studies were not only conducted at a fixed substrate concentration but also at a fixed hydrogen ion concentration. While the pH of a phosphate buffer does not change dramatically with temperature it must at the same time be conceded that the pH 7.4 buffer employed by Kalow and Lindsay (1955) does not permit measurement of maximum activity. Except at the lower temperatures therefore and then only for the usual homozygote and possibly the usual/fluoride-resistant heterozygote were the conditions employed optimal, yielding maximal velocities. There was therefore evidence that the substrate concentration necessary for enzyme saturation, and hence the Michaelis-Menten constant, the K_m , varied with temperature. Use could therefore be made of the Van't Hoff equation which relates the variation with temperature of the reaction equilibrium with the standard enthalpy (ΔH_f^O) for the reaction.

$$\Delta H_f^O = RT^2 \frac{d \log_e K_{eq}}{dT}$$

This however would only be valid if the Michaelis-Menten constant represented a true equilibrium constant. Since it is generally accepted that the reaction catalysed by cholinesterase occurs in three stages, the last two probably irreversible.



This complicates calculations based on a simple reversible chemical reaction.

However the effect of temperature on the rate constant of a reaction is given by the Arrhenius equation

$$E_a = RT^2 \frac{d \log_e k}{dT} = 2.303 \times 1.98 T^2 \frac{d \log k}{dT}$$

where E_a is the Arrhenius activation energy.

The similarity of the precise thermodynamic Van't Hoff equation to the empirical Arrhenius equation can be reconciled by appeal to the theory of absolute reaction rates which postulates that the rate of a reaction is proportional to the concentration of an activated complex which is in equilibrium with the reactants and thus governed by the usual laws of thermodynamics. It can be shown that

$$E_a = \Delta H^\ddagger + RT$$

Where ΔH^\ddagger is the enthalpy difference between the activated complex and the reactants, that is, the enthalpy of activation which is related to the other thermodynamic functions, the standard free energy (ΔF^\ddagger) and the standard entropy (ΔS^\ddagger) of activation by the equation

$$\Delta F^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$$

The energy of activation is readily calculated from the differentiated Arrhenius equation

$$E_a = \frac{4.56 T_1 T_2 \log \frac{k_2}{k_1}}{T_2 - T_1}$$

Where $\frac{k_2}{k_1}$ is the ratio of recorded activities, proportional to the rate-limiting constant, at the temperatures T_2 and T_1 ,

that is, the Q_{10} values if T_2 and T_1 are 10°C apart. Table 9 shows the activation energies so calculated from Q_{10} and Q_5 values for all six genotypes over the temperature range studied.

The Arrhenius equation.

$$\frac{d \log_e k}{dT} = \frac{E_a}{RT^2} \text{ becomes on integration}$$

$$\log_e k = \log_e A - \frac{E_a}{RT}$$

$$\text{or } \frac{E_a}{T} = -4.56 \log k + 4.56 \log A$$

Where A can be considered a constant provided the temperature range is narrow. Certainly over the range $20-30^{\circ}\text{C}$ linearity of the slopes can be accepted and A therefore considered to be a constant. Figure 19 shows the loci of variation of activation energy with temperature for all six genotypes over the temperature range examined. The slopes of the loci are then $-4.56 \log k$, that is proportional to the logarithm of the rate-limiting rate constant.

From this diagram it can be seen that the reaction catalysed by the three enzyme variants, the usual, fluoride-resistant and atypical cholinesterases have zero activation energies at 49.5°C , 39.5°C and 33°C respectively. Above these temperatures the reactions become exothermic. In addition to these points, previously recorded in earlier sections as temperatures of maximum activity, Figure 19 would indicate that transitions in the rate-limiting steps occur for the usual enzyme at 46°C , the fluoride-resistant variant at 40.5°C and the atypical isoenzyme at 41°C . The latter two transitions occur in the exothermic phase

Table 9 Activation Energies of Cholinesterase Phenotypes.
(Cal/mole/degree)

	u u E _a	u f E _a	u a E _a	f f E _a	a f E _a	a a E _a
15-20	9310	12000	9560	9310	10800	11500
20-25	12400	10900	9310	8550	7730	6890
25-30	11000	9550	9390	5930	5320	4600
30-35	9440	9440	7650	3630	2155	730
35-40	7830	7600	6320	1150	-1890	-3645
40-45	6200	5870	3770	-4120	-10680	-7860
45-50	2765	808	197	-13300	-14900	-13750
50-55	-4380	-10800	-6950	-30600	-35900	-25800
15-25	11650	11500	9620	9050	9050	9240
20-30	11650	10050	9450	7150	6550	5400
25-35	10600	9550	8600	4790	3780	2390
30-40	8870	8630	7100	2470	187	-1450
35-45	7480	6810	4940	-1495	-4490	-5680
40-50	4620	3490	2095	-8650	-10880	-10680
45-55	-810	-4610	-3240	-21750	-25150	-19100

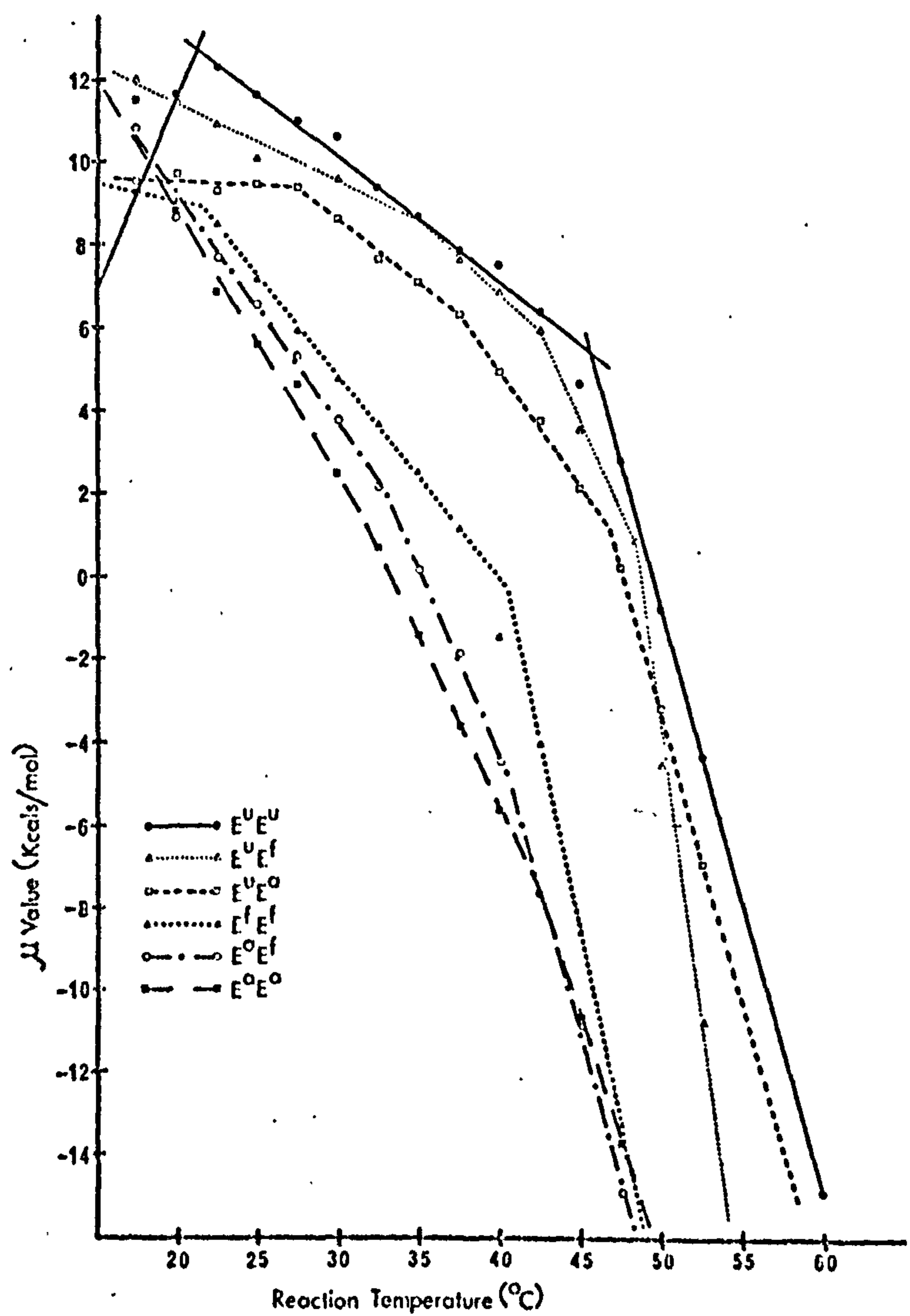


Figure 19. Variation of activation energies with temperature of the cholinesterase phenotypes.

of the reaction. Other transitional stages would appear to be indicated by change in slope of the loci at lower temperatures but there is no consistent pattern to these and it must also be conceded that at these temperatures the activities measured are comparatively low and hence least precise and reliable.

It has been assumed earlier that at the temperature of zero activation energy, reversible alterations in the protein structure occur and it is tempting to speculate that further configuration changes occur in the enzyme molecule at the transitional points but there is no basis for this in the data from which these facts were obtained. It is however valid to suggest that at different temperatures short of protein denaturation, changes do occur in the rate-limiting steps which are characteristic of the human serum cholinesterase variants.

Because of the limitations outlined earlier it is not possible to derive, nor were the experiments designed to give, any absolute thermodynamic data regarding the enzymic activation. Indeed as far as these are concerned Dixon and Webb (1958) stated

"In fact it must be admitted that remarkably little is known about any of these quantities and that such values as are given in the literature have meanings which are doubtful"

Certainly in biological work the symbol μ is used instead of E_a to signify that the quantity measured may not be as rigorously defined as the energy of activation. That aside however the data recorded in this study would indicate that under the specific experimental conditions employed

there are temperature-dependent changes in the efficiencies with which the enzymes catalyse the hydrolysis of benzoylcholine. In addition, at any specific temperature these efficiencies, as exemplified by the rate-limiting constant, of which the recorded activity is an index, also differ. Further, solely and specifically under the assay conditions employed the energies of activation at any one temperature are an index of the relative catalytic efficiencies of the variants. While these values have no absolute significance they do have a valid relative meaning and this validity is most acceptable at temperatures where activities are most accurately measured and the response to change in temperature most linear.

These last stipulations inexorably suggest the range 25-30°C and between these values the energies of activation were calculated as 11.0 Kcals for the usual homozygote, 9.55 Kcals for the usual/fluoride-resistant heterozygote, 9.39 Kcals for the usual/atypical heterozygote, 5.93 Kcals for the fluoride-resistant homozygote, 5.32 Kcals for the atypical/fluoride-resistant heterozygote and 4.6 Kcals for the atypical homozygote. These represent mean values for the sera studied as illustrated in Figures 12 and 17.

A study of these activation energies indicates that with the possible exception of the $E_1^a E_1^f$ phenotype the activation energies of the heterozygous sera are obviously not derived from a 50:50 proportional activity of the two variants. If then X is taken as the fractional activity of the usual enzyme and Y that of the fluoride-resistant variant in the heterozygote, $E_1^u E_1^f$

$$11.0 X + 5.93 Y = 9.5 \quad \text{where } X + Y = 1$$

$$\text{or } 5.93 X + 5.93 Y = 5.93$$

$$\text{Subtracting } 5.07 X = 3.57$$

$$\text{or } X = .704$$

$$\text{and } X = .296$$

Similarly for the usual/atypical heterozygote

$$11.0 X + 4.6 Y = 9.39 \quad \text{and } 4.6 X + 4.6 Y = 4.6$$

$$\text{Subtracting } 6.4 X = 4.78$$

$$\text{or } X = .748$$

$$\text{and } Y = .252$$

For the atypical/fluoride-resistant heterozygote

$$5.93 X + 4.6 Y = 5.32 \quad \text{and } 4.6 X + 4.6 Y = 4.6$$

$$\text{Subtracting } 1.33 X = 0.72$$

$$\text{or } X = .54$$

$$\text{and } Y = .46$$

The proportions of activity due to the atypical and fluoride-resistant enzymes in the $E_1^a E_1^f$ phenotype are in excellent agreement with their relative proportions in the respective usual heterozygotes.

As an independent check on the validity of these calculated proportions of activity in heterozygous sera the inhibition of the cholinesterase variants may be considered. The usual, fluoride-resistant and atypical enzymes have mean inhibitions of 80%, 65% and 20% respectively (Figures 2 and 29) in the presence of $10^{-5}M$ dibucaine under the assay conditions employed.

Considering then the $E_1^u E_1^f$ phenotype every 100 units

of activity comprises

$$70 \text{ units } E_1^u \text{ inhibited } 80\% = 14 \text{ units}$$

$$\text{and } 30 \text{ units } E_1^f \text{ inhibited } 65\% = 10.5 \text{ units.}$$

$$\text{therefore } \frac{\text{inhibited activity}}{\text{uninhibited activity}} = \frac{24.5}{100} = 75.5\% \text{ inhibition.}$$

Similarly for the $E_1^u E_1^a$ phenotype every 100 units of activity comprises

$$75 \text{ units } E_1^u \text{ inhibited } 80\% = 15 \text{ units}$$

$$\text{and } 25 \text{ units } E_1^a \text{ inhibited } 20\% = 5 \text{ units.}$$

$$\text{therefore } \frac{\text{inhibited activity}}{\text{uninhibited activity}} = \frac{20}{100} = 20\% \text{ inhibition.}$$

For the $E_1^a E_1^f$ phenotype every 100 units of activity comprises

$$46 \text{ units } E_1^a \text{ inhibited } 20\% = 9.2 \text{ units}$$

$$\text{and } 54 \text{ units } E_1^f \text{ inhibited } 65\% = 35.1 \text{ units}$$

$$\text{therefore } \frac{\text{inhibited activity}}{\text{uninhibited activity}} = \frac{44.3}{100} = 44.3\% \text{ inhibition}$$

These calculated inhibitions or dibucaine numbers are in excellent agreement with the mean values found for the heterozygous phenotypes (Figures 2 and 29).

By the same procedure using mean fluoride inhibitions of 60%, 37% and 24% for phenotypes $E_1^u E_1^u$, $E_1^f E_1^f$ and $E_1^a E_1^a$ respectively the calculated mean inhibitions for the heterozygotes $E_1^u E_1^f$, $E_1^u E_1^a$ and $E_1^a E_1^f$ are 53%, 51% and 31%, figures which are in good agreement with the values determined experimentally. The same agreement is found between the calculated and experimentally determined figures for chloride and succinyl-dicholine inhibition (Table 10).

Table 10 Inhibitor Numbers experimentally determined and calculated from thermodynamid data.

	Relative Activities	DN	FN	C1 N	SN
$E_1^u E_1^u$	Experimentally determined	80	62	10	90
	Equating μ values	-	-	-	-
		-	-	-	-
$E_1^u E_1^f$	Experimentally determined	75	52	20	84
	Equating μ values	75.5	53.5	13	85
	50:50 μ values	75	52	16	84.5
		62	51	25	65
$E_1^u E_1^a$	Experimentally determined	65.5	52	21	71.5
	Equating μ values	75:25	52	21	71.5
	50:50 μ values	70.5:29.5	50.5	22.5	68.5
		66	34	27	74
$E_1^f E_1^f$	Experimentally determined	-	-	-	-
	Equating μ values	-	-	-	-
	50:50 μ values	-	-	-	-
		49	33	38	48
$E_1^a E_1^f$	Experimentally determined	45	28.5	39.5	47
	Equating μ values	46:54	29	39	48
	50:50 μ values	46.5	29	39	48
		21	22	54	15
$E_1^a E_1^a$	Experimentally determined	-	-	-	-
		-	-	-	-
		-	-	-	-

It is a matter of common observation that the inhibitor numbers of heterozygous sera do not occupy a medial position between the homozygous phenotypes. For instance the mean dibucaine numbers for the usual and atypical enzymes are 80 and 20 respectively while that of the heterozygote is about 62. As discussed in greater detail later (page 86 et seq.) in the heterozygote this could be due to an unequal rate of synthesis of the two variant enzymes, to unequal enzymic efficiencies or a combination of both. However the foregoing data is independent of the absolute activity, that is, the quantity of enzyme, but solely reflects the catalytic efficiency of the enzyme in the same way that the turnover number does. The foregoing calculations in equating enzymic efficiencies (μ values) does not rule out the possibility of unequal rates of synthesis of the variants, but only show that in the heterozygote the activities due to the enzymic species are not equal.

However if one assumes equal molecular proportions of the usual and fluoride-resistant enzymes in the heterozygote the percentage activity due to the usual variant would be

$$\frac{100 \times 11.0}{11.0 + 5.93} = \frac{1100}{16.93} = 65\%$$

By similar arithmetic it can be shown that on an equimolecular basis, the proportions of activity in the $E_1^u E_1^a$ and $E_1^a E_1^f$ heterozygotes are 71:29 and 44:56 respectively. The inhibitions calculated from these proportions are shown in Table 10 and if anything are closer to the experimentally determined means than those calculated from equating the μ values.

The difference between the two sets of calculated proportional activities in the heterozygotes are obviously not significant and can readily be appreciated when it is considered that all the values involved are mean figures and there are cogent reasons to believe that the currently accepted variants are not homogeneous (p. 90 et seq.). Certainly the foregoing indicates that there is no need to postulate a quantitative difference in the rates of synthesis of the enzyme variants in the heterozygous state; all the observed experimental facts are entirely resolved on qualitative differences.

Confirmation of some of the experimental and derived data obtained in this study is to be found in the literature. Thus Kalow and Lindsay reported an activation energy of the usual homozygote to be 11.2 Kcals/degree/mole which is in good agreement with that recorded herein. The literature does not appear to contain values for the activation energies of the other cholinesterase variants. In the classical studies of Liddell, Lehman, Davies and Sharih (1962) the usual and atypical enzymes in heterozygous sera were separated by chromatography on diethyl amino-ethyl cellulose columns and by paper electrophoresis at pH 9.7. Determination of the cholinesterase activity in the chromatography eluate fractions gave a usual to atypical ratio of 69:31 and from paper strip elution of 71:29. These values are in excellent agreement with those obtained on an equimolecular basis in this study. No other such ratios appear to have been determined and Lehmann and co-workers did not seek confirmation of their activity ratios by

calculating the inhibitions of the heterozygote from those of the homozygotes.

2. (c) Inhibition of Benzoylcholine Hydrolysis.

Inhibition of Benzoylcholine Hydrolysis

In routine analysis of specimens referred from "suxamethonium apnoeas" and in preliminary studies, the inhibitions by 10 μ M dibucaine (Kalow and Genest, 1957) and 50 μ M sodium fluoride (Harris and Whittaker, 1961) of the hydrolysis of a 50 μ M benzoylcholine substrate in phosphate buffer, pH 7.4 and at 25°C (Kalow and Lindsay, 1955) have been found necessary and sufficient for precise phenotype identification. At an early stage in these investigations the inhibition by 500 mM sodium chloride (Whittaker, 1968b) was also included in the hope that correlations of the three inhibitors might yield further evidence to substantiate the claim (Whittaker 1968c) that its use revealed new phenotypes.

It seemed reasonable that, in the specific clinical setting under investigation, that of suxamethonium apnoea, use of the causative agent, succinylcholine, as a differentiating substrate or inhibitor would have potential advantages. The investigation of succinylcholine and its sulphur analogue as substrates is detailed later (p.103 et seq.).

In the light of modern theories of neuro-muscular transmission, the classification of muscle-relaxant drugs becomes increasingly complex. In the classic picture which satisfied elementary stereochemical, pharmacological and physiological precepts, such drugs were separated into two categories, depolarising and non-depolarising. The former were long, thin molecules which achieved their effect by depolarising the motor end plate in the same way as acetylcholine. Indeed high doses of acetylcholine itself produce blockade and

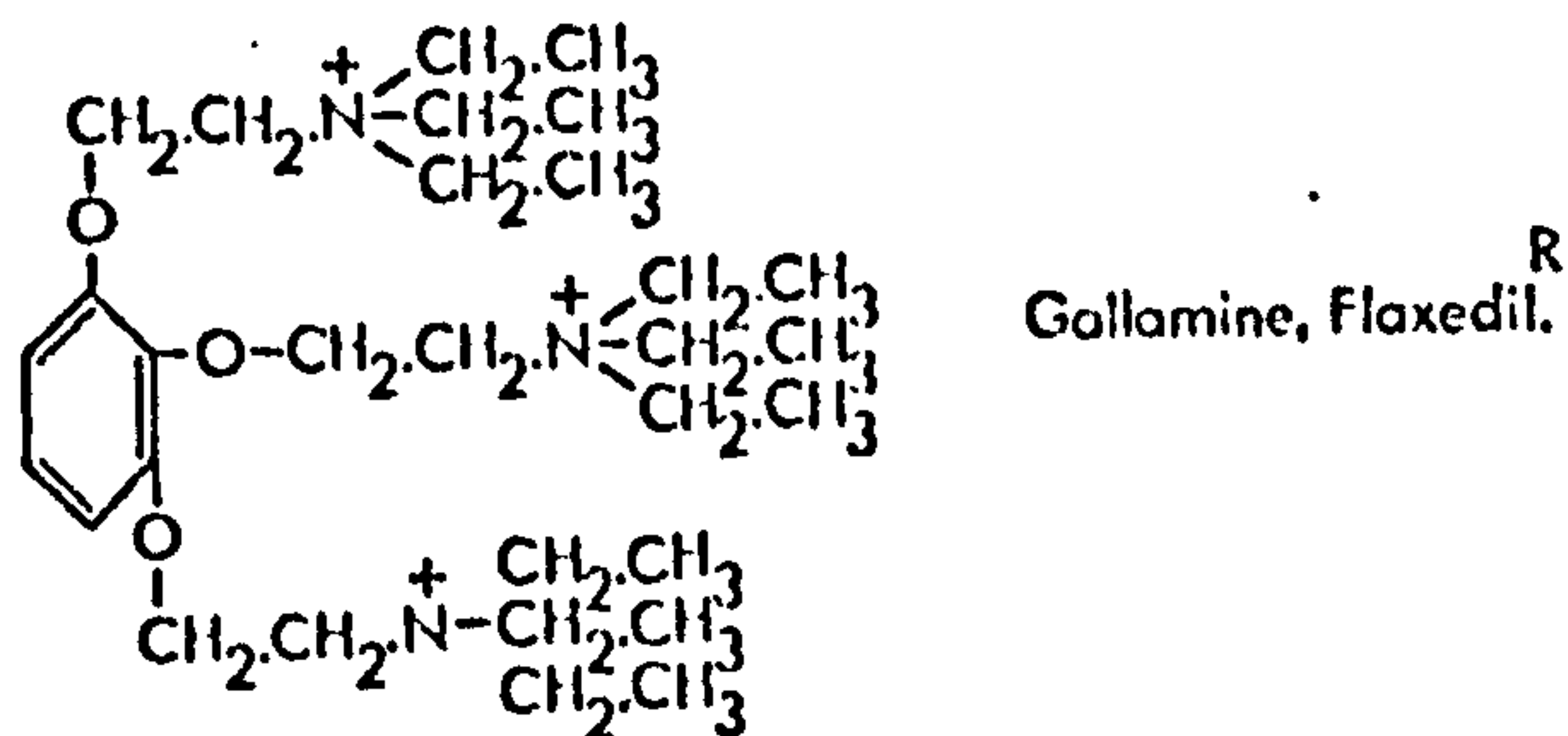
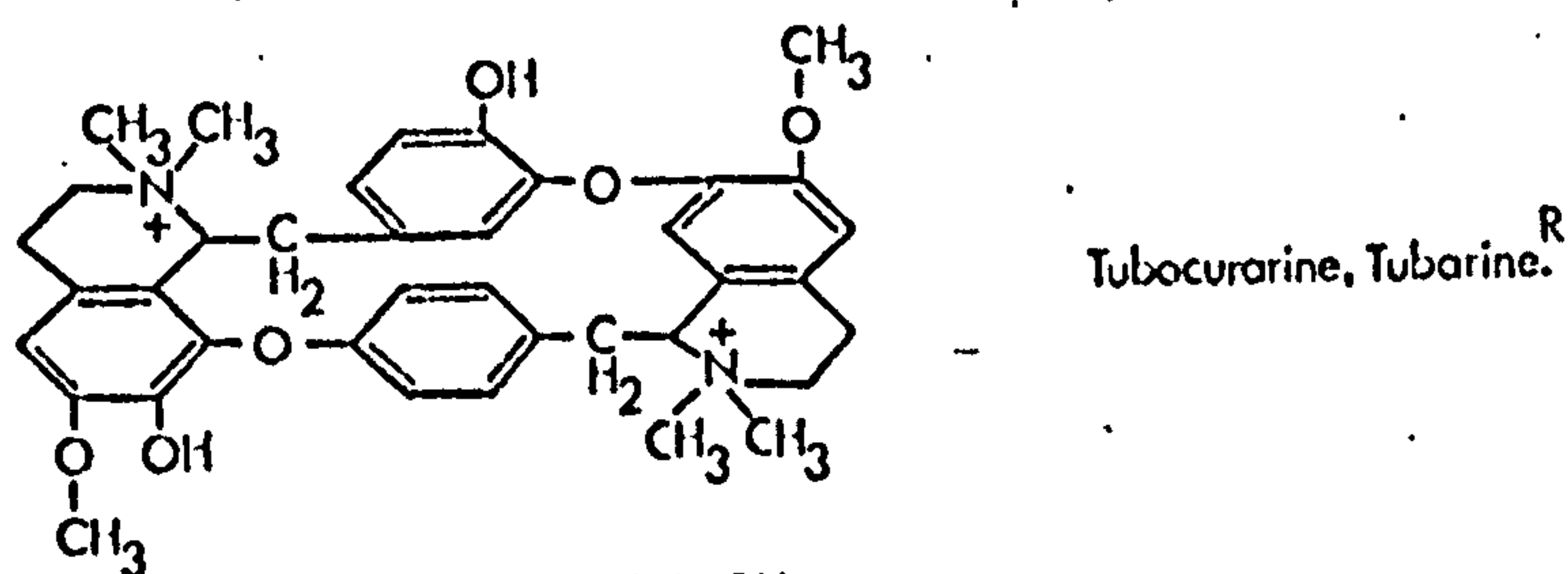
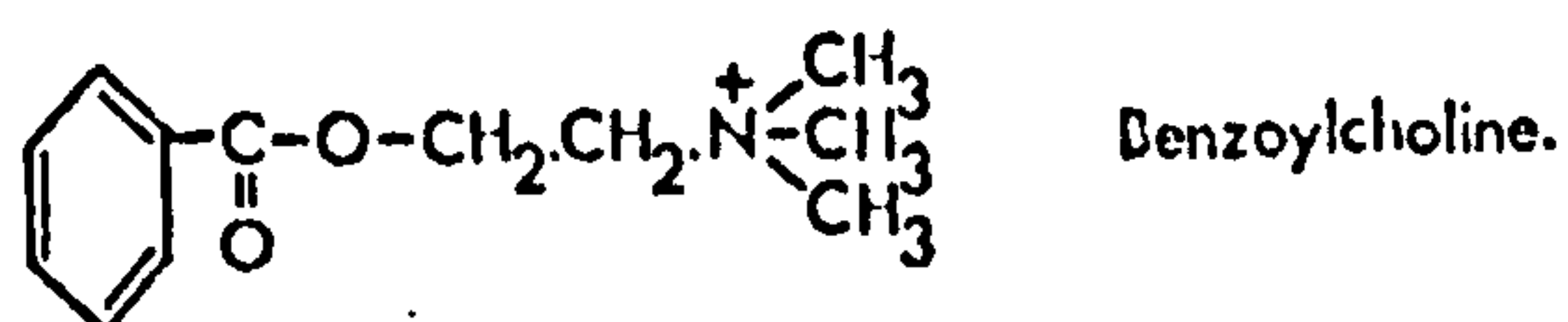
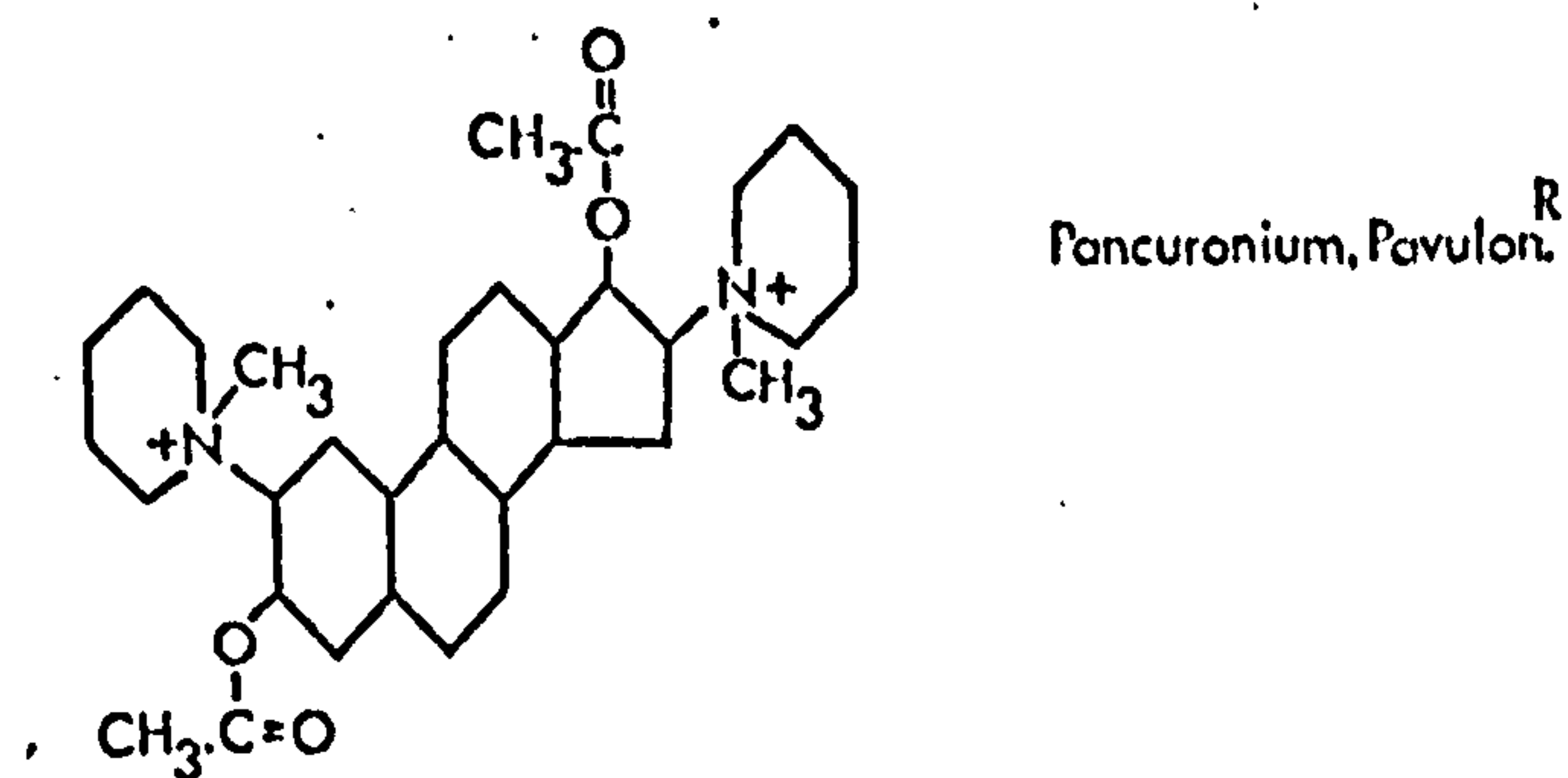


Figure 20. Structural formulae of some substrates and inhibitors of cholinesterase. Other examples are illustrated in Figure 1 indicating the affinity of cholinesterase for compounds containing a positively charged nitrogen atom particularly when linked to methyl or ethyl groups.

through this so do the anti-acetylcholinesterases. These are therefore classed as depolarising agents. The non-depolarising relaxants were bulky molecules which acted post-synaptically by occupying the receptor sites for acetylcholine. Succinyldicholine and tubocurarine are the typical examples of depolarising and non-depolarising muscle relaxants respectively and their structural formulae are shown in Figure 20.

Pancuronium bromide (Pavulon) is a recently-introduced non-depolarising muscle relaxant. The structural formula (Figure 20) shows it to be steroidal with two methyl pyridine and two acetyl residues attached. It is interesting to note that in both instances the ester oxygens are separated by two carbon atoms from the quaternary nitrogens. Pharmacologically the neuromuscular block produced by pancuronium is of curariform type and stereochemically the molecule is typically non-depolarising. However the presence of the methylated quaternary nitrogens and acetyl groups suggested possible anticholinesterase potential and this latter aspect was accordingly investigated.

Instrumentation, Methods and Materials

Instrumentation and methods were as described in the previous section (page 42). All assays were carried out at 25°C unless otherwise stated.

In addition to the reagents previously employed (page 43) succinyldicholine chloride dihydrate was obtained from BDH Chemicals, Pancuronium bromide (Pavulon) from Organon Laboratories, tubocurarine chloride (Tubarine) from Burroughs Wellcome and gallamine triethiodide (Flaxedil)

from May and Baker Ltd. Aqueous solutions of these inhibitors were made up to different concentrations and 1 ml aliquots employed as inhibitors in the assay scheme detailed earlier (page 44).

Sera from six phenotypes, 100 usual homozygotes $E_1^u E_1^u$, 25 usual/fluoride-resistant heterozygotes $E_1^u E_1^f$, 61 usual/atypical heterozygotes $E_1^u E_1^a$, 3 fluoride-resistant homozygotes $E_1^f E_1^f$, 12 atypical/fluoride-resistant heterozygotes $E_1^a E_1^f$ and 20 atypical homozygotes $E_1^a E_1^a$ were used in defining the phenotype ranges and in comparing inhibitor numbers.

Experimental Results

At a benzoylcholine concentration of 50 μ M the succinyldicholine concentration in the reaction mixture was varied from 5 μ M to 100 mM. The inhibition curves obtained for the six commonly-recognised phenotypes are shown in Figure 21. These follow the typical sigmoid pattern. It was evident that maximum differentiation between the phenotypes was obtained at a 1 mM concentration of succinyldicholine in the reaction mixture. Preincubation of the reaction mixture without benzoylcholine, that is, buffered dilute serum in the presence of 1 mM succinyldicholine for periods up to one hour did not alter the percentage inhibition significantly.

By employing a 4 mM aqueous solution of succinyldicholine (397 mg succinyldicholine chloride dihydrate per litre) in the assay scheme outlined earlier the percentage inhibition (Scoline Number) was determined simultaneously with dibucaine, fluoride and chloride numbers on the sera of 221 individuals as detailed above. The values obtained

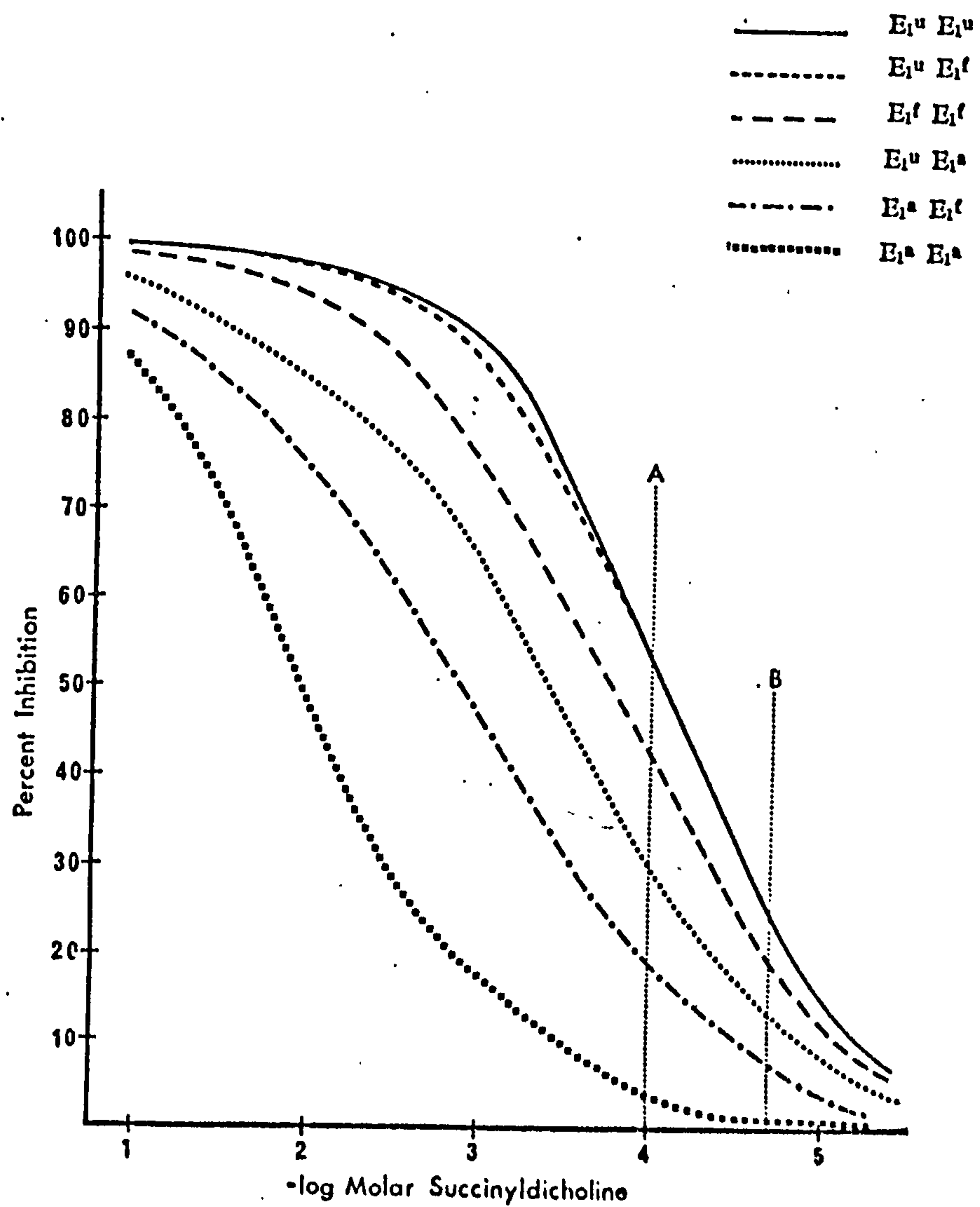


Figure 21. Inhibition of serum cholinesterase activities by succinylcholine in the assay procedure of Kalow and Lindsay (1955) at 25°C. Theoretical concentrations in the blood plasma following injection of 100 mg succinylcholine are shown initially at A and after 20-30 minutes at B.

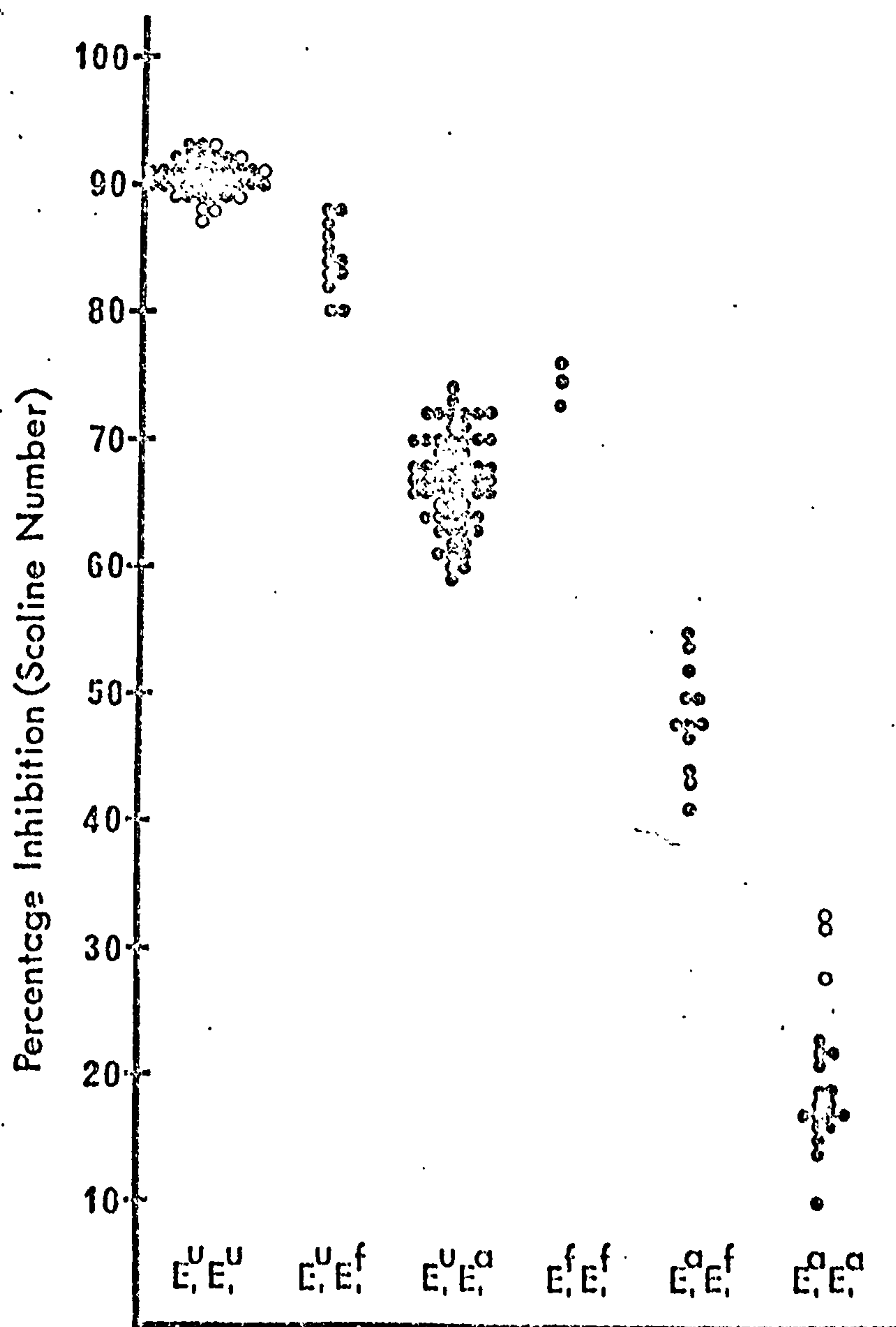


Figure 22. Cholinesterase phenotype inhibitions by mM succinylcholine in the presence of 50 μM benzoylcholine in phosphate buffer, pH 7.4 at 25°C.

for succinylcholine inhibition in this survey over a period of two years are shown in Figure 22. In this illustration, the closed circles for the usual phenotype each represent three sera, the open circles, one specimen. Three anomalous values obtained from serum samples of two sisters were assigned to the atypical homozygote group where they are shown as open circles.

Figures 23, 24 and 25 illustrate the correlations between the distribution of Scoline numbers and dibucaine, fluoride and chloride numbers respectively. The remaining correlations are also illustrated between dibucaine and fluoride numbers in Figure 26, between dibucaine and chloride numbers in Figure 27 and between fluoride and chloride numbers in Figure 28. A point in the centre of a symbol in these diagrams indicates two sera with identical numbers.

The phenotype ranges for all four inhibitors derived from this study are shown in Figure 29, (King, 1974). It should perhaps be emphasised that in this study where a result was doubtful or of a controversial nature it was confirmed by repeated estimations.

Pavulon is a solution of 2 mg pancuronium dibromide per ml that is $2.63 \times 10^{-3} \text{M}$. A dilution therefore of 1 ml Pavulon and 2.29 ml of water gives a solution $8 \times 10^{-4} \text{M}$ with respect to the drug and 1 ml of this solution in a final assay mixture of 4 ml gives a reaction concentration of $2 \times 10^{-4} \text{M}$. Dilutions to give reaction concentrations down to $2 \times 10^{-8} \text{M}$ were prepared and the inhibition of benzoylcholine hydrolysis by a variety of phenotype sera studied. Typical results obtained are illustrated in

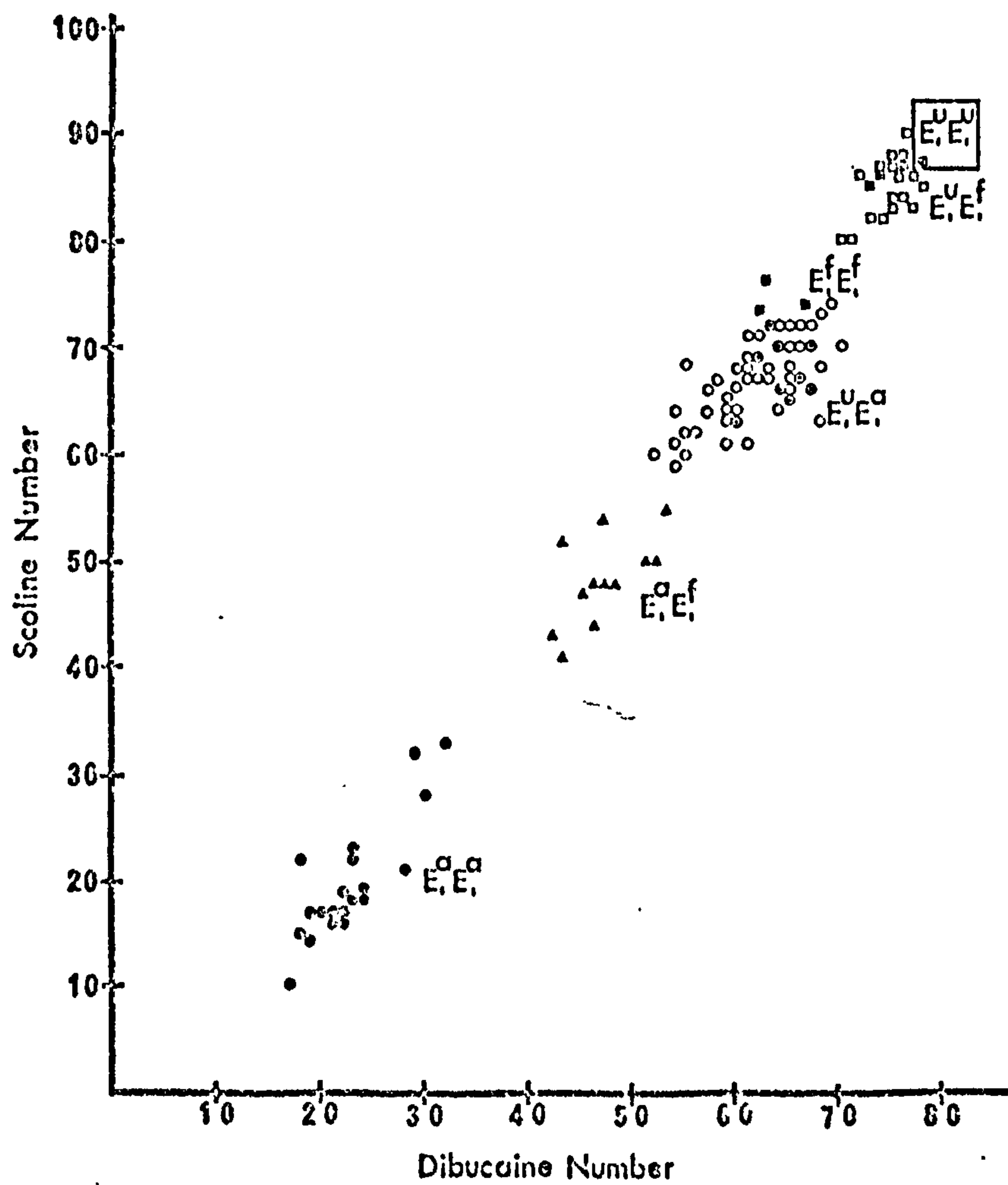


Figure 23. Correlation of Scoline Numbers and Dibucaine Numbers for the cholinesterase phenotypes.

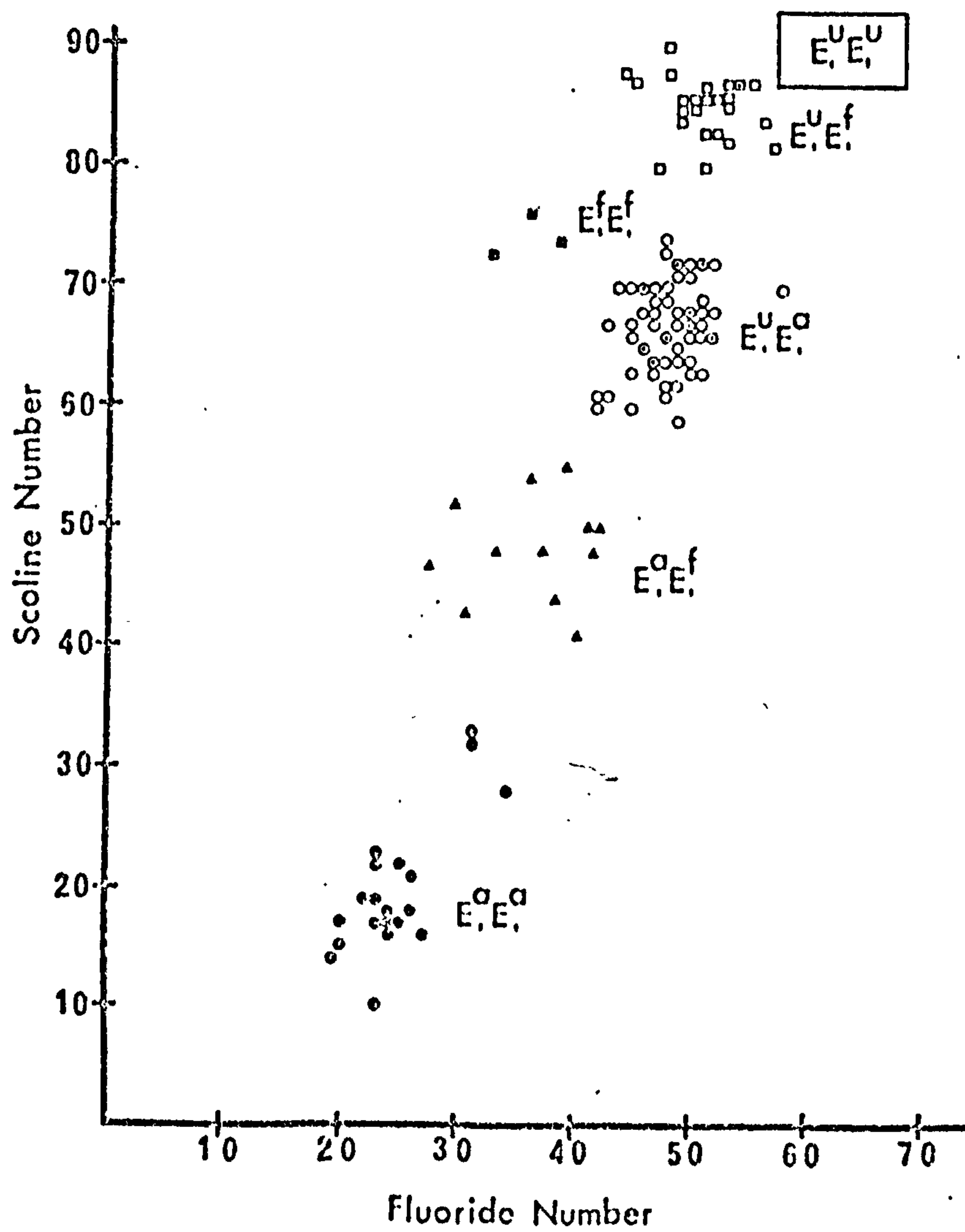


Figure 24. Correlation of Scoline Numbers and Fluoride Numbers for the cholinesterase phenotypes.

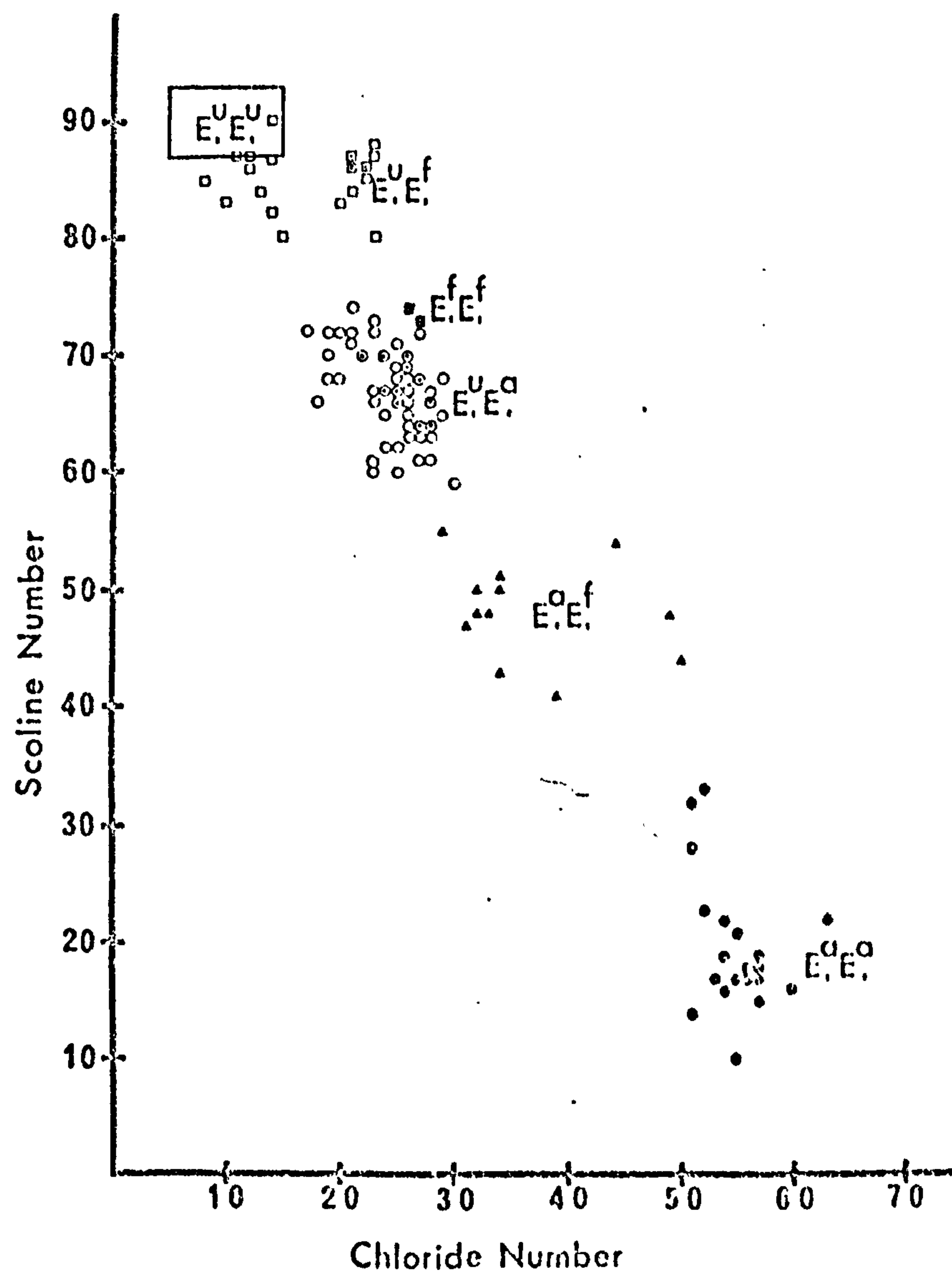


Figure 25. Correlation of Scoline Numbers and Chloride Numbers for the cholinesterase phenotypes.

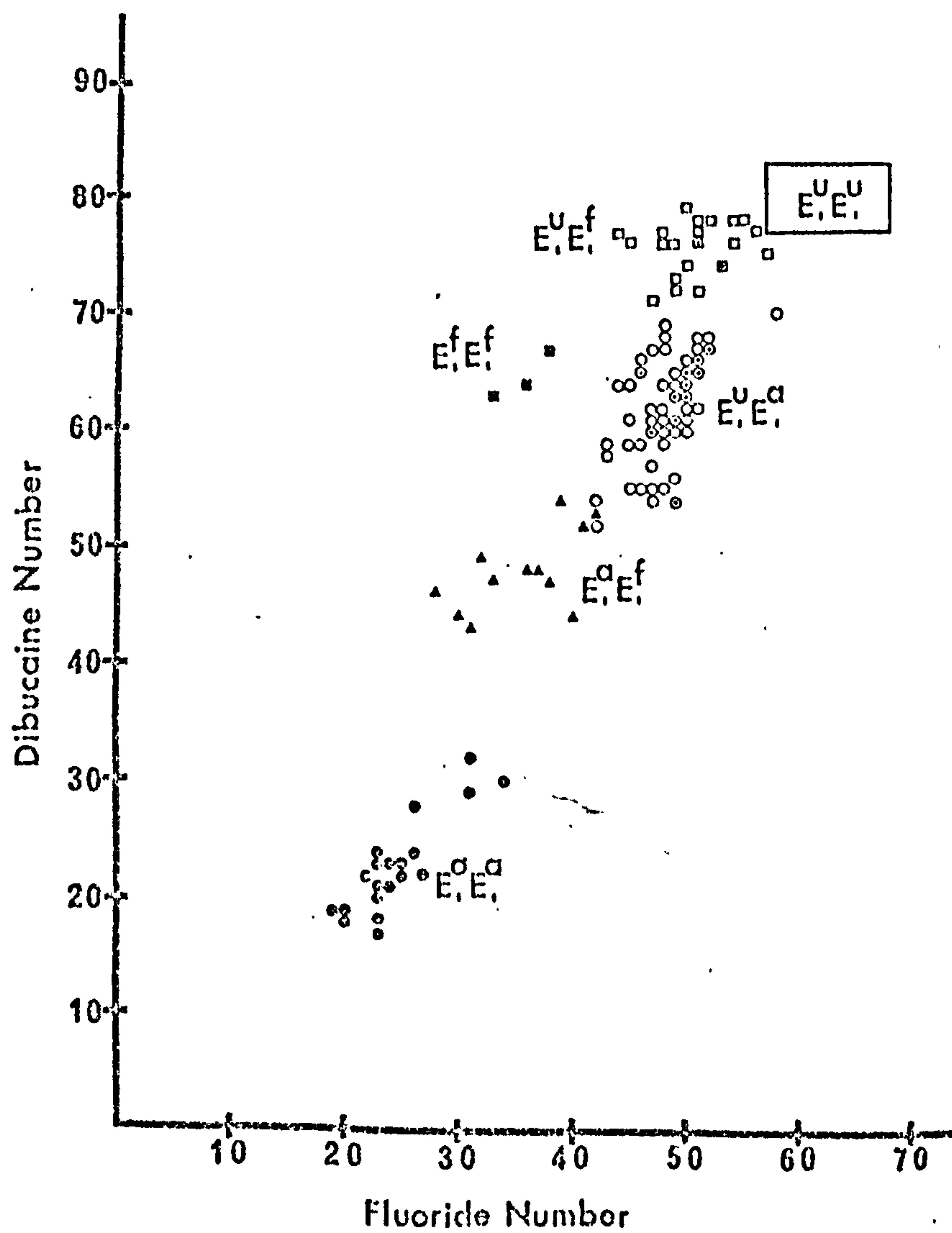


Figure 26. Correlation of Dibucaine Numbers and Fluoride Numbers for the cholinesterase phenotypes.

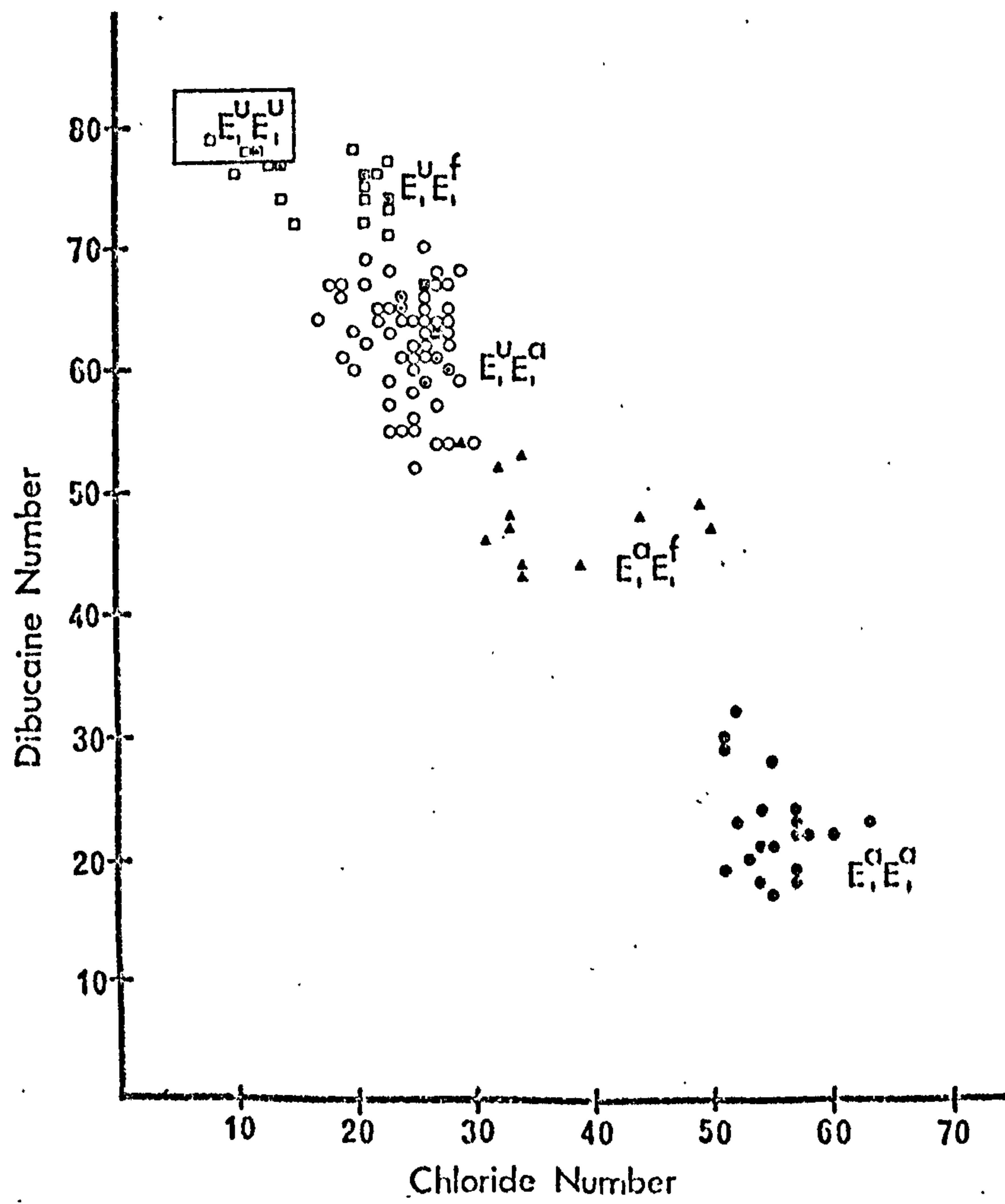


Figure 27. Correlation of Dibucaine Numbers and Chloride Numbers for the cholinesterase phenotypes.

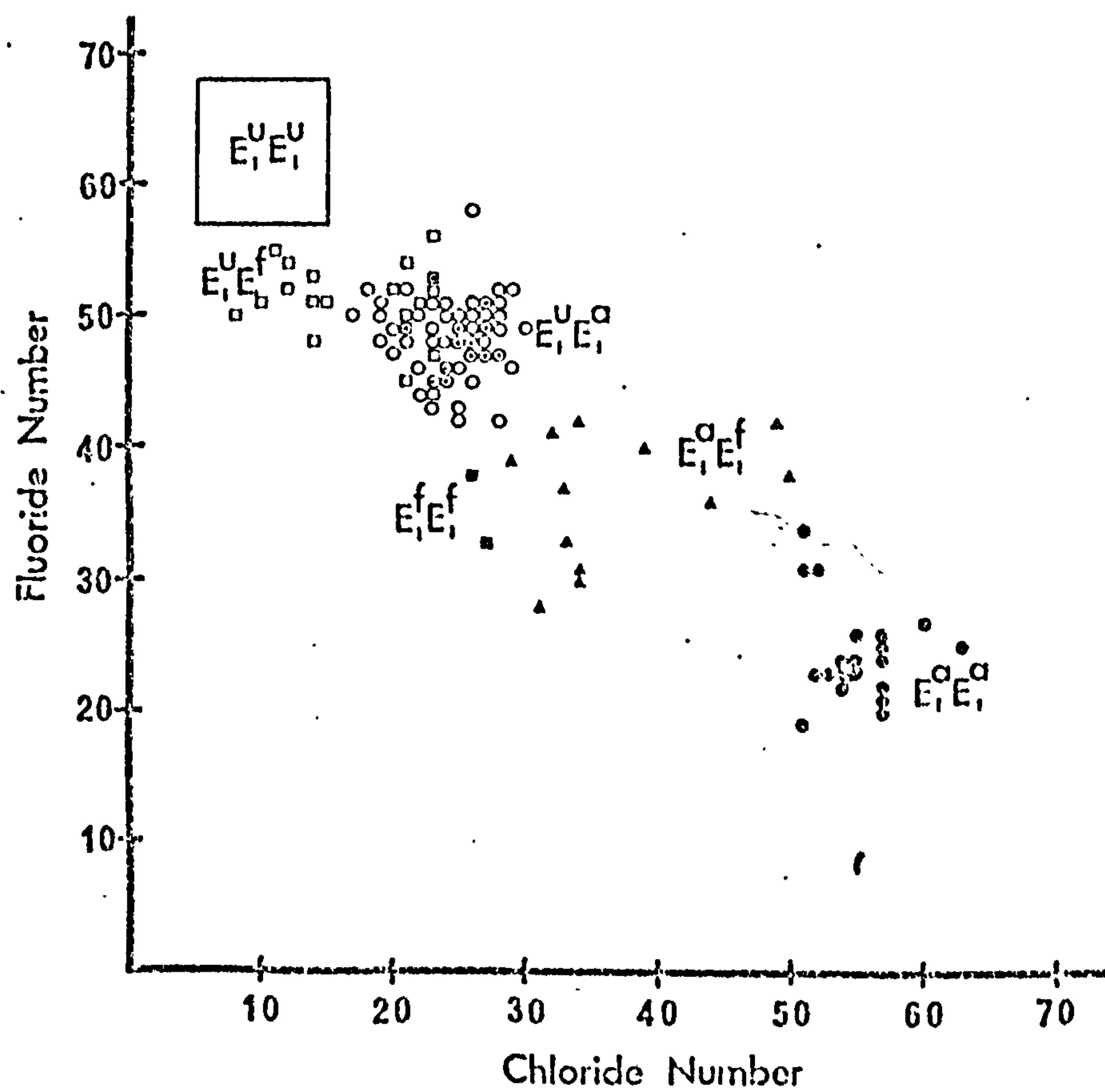


Figure 28. Correlation of Fluoride Numbers and Chloride Numbers for the cholinesterase phenotypes.

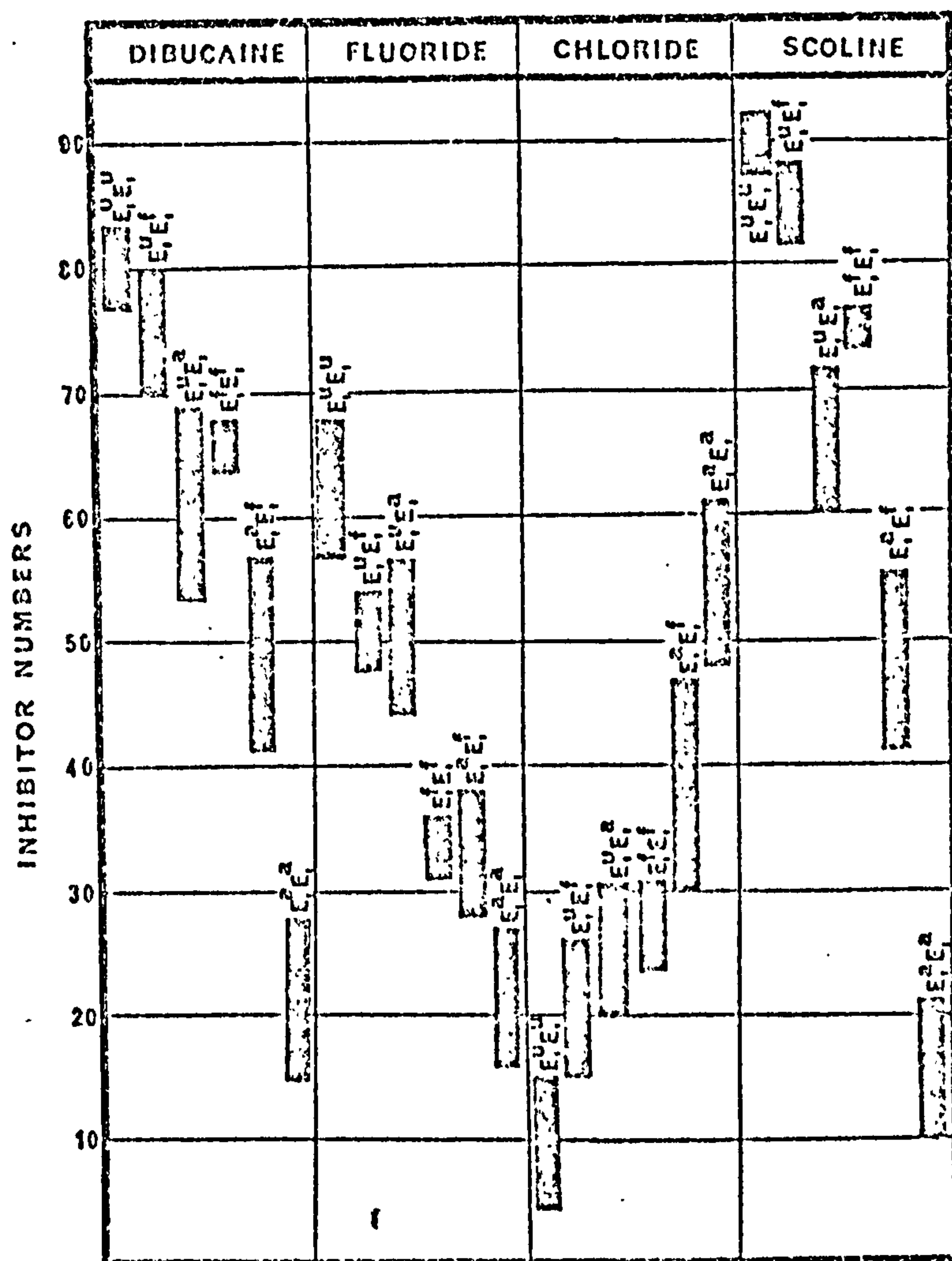


Figure 29. Phenotype ranges of inhibitor numbers (percentage inhibition) by 10 μ M dibucaine, 50 μ M sodium fluoride, 500 mM sodium chloride, mM succinylcholine in the presence of 50 μ M benzoylcholine in phosphate buffer pH 7.4 and at 25°C.

Figure 30.

Similarly for tubocurarine and gallamine, concentrations of the drugs to give reaction concentrations from 10^{-3} M to 10^{-5} M were prepared and the inhibitions recorded. These are shown in Figure 31.

Discussion

Kalow and Genest (1957) made the observation that the dibucaine number of an individual remains remarkably constant even when the serum cholinesterase activity changes. This fact implies a constant proportion of the two enzyme variants in any particular heterozygote. Furthermore Kalow and Staron (1957) reported that even when the cholinesterase activity of a serum sample decreased upon storage, the dibucaine number remained unaltered for as long as it could be determined. This fact implies an equal in vitro stability of the cholinesterase variants. These observations of Kalow and co-workers only applied to dibucaine inhibition and only knowingly to the usual and atypical variants but the in vivo constancy of percentage inhibition for all phenotypes and inhibitors studied has been confirmed in the present study except in one set of circumstances (page 123). While it has been noted that the usual and atypical isoenzymes are apparently equally stable to storage the fluoride-resistant variant would appear to be somewhat less so under these conditions.

The finding that preincubation of dilute serum with mM succinylcholine for one hour did not alter the percentage inhibition is in agreement with Kalow (1959a) who showed that the usual and atypical enzymes at this

concentration of succinylcholine hydrolysed about 80 μmol and less than 5 μmol per minute per litre respectively at 30°C. This is equivalent to hydrolysis of 96 nmol and less than 6 nmol per hour by 20 μl of serum which from Figure 21 would not result in any significant change in inhibition. The figures given by Goedde, Held and Altland (1968) for the spontaneous hydrolysis of succinylcholine are of the same order as that for the atypical enzyme. These workers studied the inhibition by succinylcholine of benzoylcholine hydrolysis by the usual and atypical enzymes and the curves they obtained are almost identical with those reported here (Figure 21). The literature does not appear to contain any similar study relating to the other cholinesterase phenotypes.

The technical advantages to the use of succinylcholine in differentiating the serum cholinesterase variants are limited. Less skill is required in preparing the reagent. For a litre of solution an error of a few milligrams in the required 397 mg of succinylcholine chloride dihydrate will not significantly alter the results, whereas with dibucaine and sodium fluoride, 13.7 mg and 8.4 mg respectively must be accurately weighed for one litre of solution. This robustness of the reagent does not extend to storage however where it was found to be no more stable than the dibucaine solution and, not unexpectedly, much less than the fluoride and chloride reagents.

Lehmann and Liddell (1969) indicated that about one in 3,200 of the usual homozygote population is found to be moderately sensitive to suxamethonium and that this accounted

for 37% of suxamethonium apnoeas. It had been hoped, rather than confidently expected, that employing the actual causative agent as inhibitor might demonstrate a new phenotype and permit differentiation of sensitive usual homozygotes. Eleven such individuals were tested during this survey and did not exhibit any difference to succinylcholine inhibition from the other usual homozygotes.

There is nevertheless an undoubted advantage in interpreting results from succinylcholine inhibition in that the difference between the means of the usual homozygotes (90%) and the atypical homozygotes (18%) is greater than that obtained with dibucaine. Figure 23 however indicates that there is a close correlation between the two inhibitor numbers.

It is only a comparison of Figures 24 and 26 which shows any divergence of interpretation. Three of the specimens classified as $E_1^a E_1^f$ by Scoline and fluoride number combination (Figure 24) would probably be classed as $E_1^u E_1^a$ by dibucaine and fluoride numbers (Figure 26). It is not possible by family studies to decide this matter, although in one case involved in an apnoeic episode, it may be possible at a later date if there are any children. The wide range of inhibitor numbers which is found particularly in heterozygotes, has been the cause of much speculation and some experimental work.

Apart from experimental error, there would appear to be two possible causes, either the synthesis of enzyme due to each gene is separately controlled, that is, the proportions of variants in heterozygous sera varies, or

there are numerous cholinesterase variants with different enzymic properties, mainly turnover number, but also possibly, relative stabilities.

With regard to the experimental error, the assay of cholinesterase activity in these studies has an error of $\pm 2.4\%$ and replicate estimates of dibucaine number around 20 and 80 have maximum variations of ± 3 and ± 1 respectively. The scatter of results due to experimental error can be readily calculated. Assuming a higher, rounded figure of 3% for the coefficient of variation the ratio is independent of the actual level of cholinesterase activity which again for ease may be taken as the rounded figure of 1000 mU/ml. Then for the usual homozygote with 80% inhibition by dibucaine the outside limits of error are:

$$\frac{20\% \text{ activity } \pm \text{C.V.}}{100\% \text{ activity } \mp \text{C.V.}} \quad \text{or} \quad \frac{206}{970} \quad \text{to} \quad \frac{194}{1030}$$

that is, 79 to 81

For the atypical homozygote inhibited 20% the limits of dibucaine number are:

$$\frac{80\% \text{ activity } \pm \text{C.V.}}{100\% \text{ activity } \mp \text{C.V.}} \quad \text{or} \quad \frac{824}{970} \quad \text{to} \quad \frac{776}{1030}$$

that is, 15 to 25

The fluoride-resistant homozygote with 65% inhibition by dibucaine will have limits:

$$\frac{35\% \text{ activity } \pm \text{C.V.}}{100\% \text{ activity } \mp \text{C.V.}} \quad \text{or} \quad \frac{360.5}{970} \quad \text{to} \quad \frac{339.5}{1030}$$

that is 63 to 67

The theoretical limits of dibucaine number due to experimental error so calculated and the actual ranges encountered (Figure 29) are shown in Table 11. The conclusion to be

Table 11

Dibucaine Numbers

Phenotype	Theoretical Range	Experimental Range
$E_1^u E_1^u$	80 ± 1	77 - 83
$E_1^u E_1^f$	75 ± 1.5	70 - 80
$E_1^u E_1^a$	60 ± 2.5	54 - 69
$E_1^f E_1^f$	65 ± 2	64 - 67
$E_1^a E_1^f$	50 ± 3	41 - 56
$E_1^a E_1^a$	20 ± 5	15 - 28

derived from this is that, apart from the fluoride-resistant homozygote where only three values have been obtained to establish the actual range, the ranges of inhibitor numbers are real and not a result of lack of precision in the assay. Accuracy of activity measurement does not enter into these calculations since the inhibitor number is independent of the absolute level of activity. Accordingly the scatter of results due to experimental error in other inhibitor numbers follows the same pattern, being least (± 1) at highest percentage inhibitions (80%) and greatest (± 5) at lowest inhibitions (20%) with intermediate gradations.

That different amounts of enzyme are synthesised through each gene was postulated by Kalow and Staron (1957) and Kalow (1959a). These workers noted that the distribution curve of dibucaine numbers in what they termed the intermediate group was negatively skewed, that is, the tail extended towards the lower numbers, and also that the position was asymmetric between the atypical and usual homozygote distributions. This observation has been amply confirmed in the present study (Figure 29). Kalow and Staron (1957) then mixed equal volumes of usual homozygous and atypical homozygous sera and both by calculation from means and by experiment found the dibucaine number to be 61.5. When the mixture contained two parts of usual serum to one part of atypical serum the percentage dibucaine inhibition was 68.8. By this means they considered it possible to translate each dibucaine number of the intermediate group into a "percentage of atypical esterase" and found the mean to be 50.2 with a standard deviation of 9.0.

The distribution curve obtained from these calculations was superimposed on the numbers determined experimentally but it was found that "the fit of the data to this curve is imperfect". This is not surprising since from the data presented by Kalow and Staron (1957) and enlarged later Kalow (1959b) the series contained sera from atypical/fluoride-resistant heterozygotes, the existence of which was not known at that time (Lehmann, Silk, Harris and Whittaker, 1960) and the existence of which they were endeavouring to disprove.

The basic argument is unsupportable since in mixing equal parts of the two sera they assumed that they were mixing equal amounts of the two enzymes. In other words the argument was based on the premise that the genes in the atypical homozygote brought about the synthesis of enzyme molecules at the same rate as the genes in the usual homozygote and this was then used to prove that the genes in each individual mediated different rates of synthesis. On the contrary if the fact that the mean value obtained from their calculations of 50.2 is accepted as proof that the original assumption was correct (and the concordance with the values obtained in this study from thermodynamic data lends confirmation) then this in turn provides evidence that there is no genetic control of the number of enzyme molecules only of the type of enzyme molecule.

Despite this apparently unappreciated argument in favour of genetic control of qualitative but not quantitative enzyme synthesis, Kalow (1959a) presented interpretation of some of the data of Kalow and Staron (1957) "assuming

genetical control of type and activity of cholinesterase" but concluded "while it is necessary to postulate a control of activity, the observed values do not conform rigidly". A study of the presentation reveals that even with an unexplained juggling of supposed proportions of activity due to each gene any semblance of conformity is only achieved in two members of the second generation and none at all in the third generation.

Kalow (1959b) concluded his discussion:

"However the total blood of a person contains nearly a kilogram of haemoglobin and perhaps 20 mg of serum cholinesterase: the assumption is inescapable that the levels of proteins are under genetical control".

This argument could readily be reworded to the alternative proposition and say that in the blood of adult humans there occur more than 100 variants of haemoglobin and to date only four of serum cholinesterase: the assumption is inescapable that numerous cholinesterase variants are as yet unidentified.

Certainly among the "silent gene" homozygotes heterogeneity has been conclusively demonstrated (Goedde and Altland, 1968; Rubinstein, Dietz, Hodges, Lubrano and Czebotor, 1970) and either explicitly or implicitly the presence of subtypes has been suggested by chloride inhibition (Whittaker, 1968b; King and Dixon, 1970) by the effect of n-butyl alcohol (Whittaker, 1968a) or by substrate affinities (Irwin and Hein, 1966).

The three sera of controversial phenotype (Figures 24 and 26) can readily be explained on either premise but the concept that there may be numerous subtypes of cholinesterase gains support from the three anomalous results from two

sisters who were classified as atypical homozygotes despite the unusually high inhibition values (Figure 22). This in turn could suggest that there is not a single entity at present classed as "usual", "atypical" or "fluoride-resistant" enzyme but that each of these designations embraces a heterogeneous population. There may well be therefore a continuous range of cholinesterases within that which is presently termed a phenotype and characterised by the enzymic properties of the molecular species.

Unlike succinylcholine, pancuronium is reputedly not hydrolysed by cholinesterase and its molecular structure would tend to support this. Under these circumstances any inhibition observed with pancuronium would not be an index of its rate of enzymic hydrolysis but of a simple blocking of the active site. From Figure 30 it can be seen that the effect of pancuronium on benzoylcholine hydrolysis is in general very similar to that of succinylcholine. Pancuronium could therefore readily be employed for differentiating the allelic variants but for the lack of contrast in effect between the usual and fluoride-resistant enzymes.

The plasma levels of most injected substances show a biphasic reduction, the first rapid followed by a more gradual decline. The half-life of the first phase is generally of the order 4-7 minutes. Five circulation times or about $7\frac{1}{2}$ minutes is usually accepted as necessary for even distribution of injected substances throughout the plasma and therefore it is inferred that the first rapid phase represents a transfer from the plasma to the interstitial fluid (Kalow, 1959b). Taking the circulating plasma

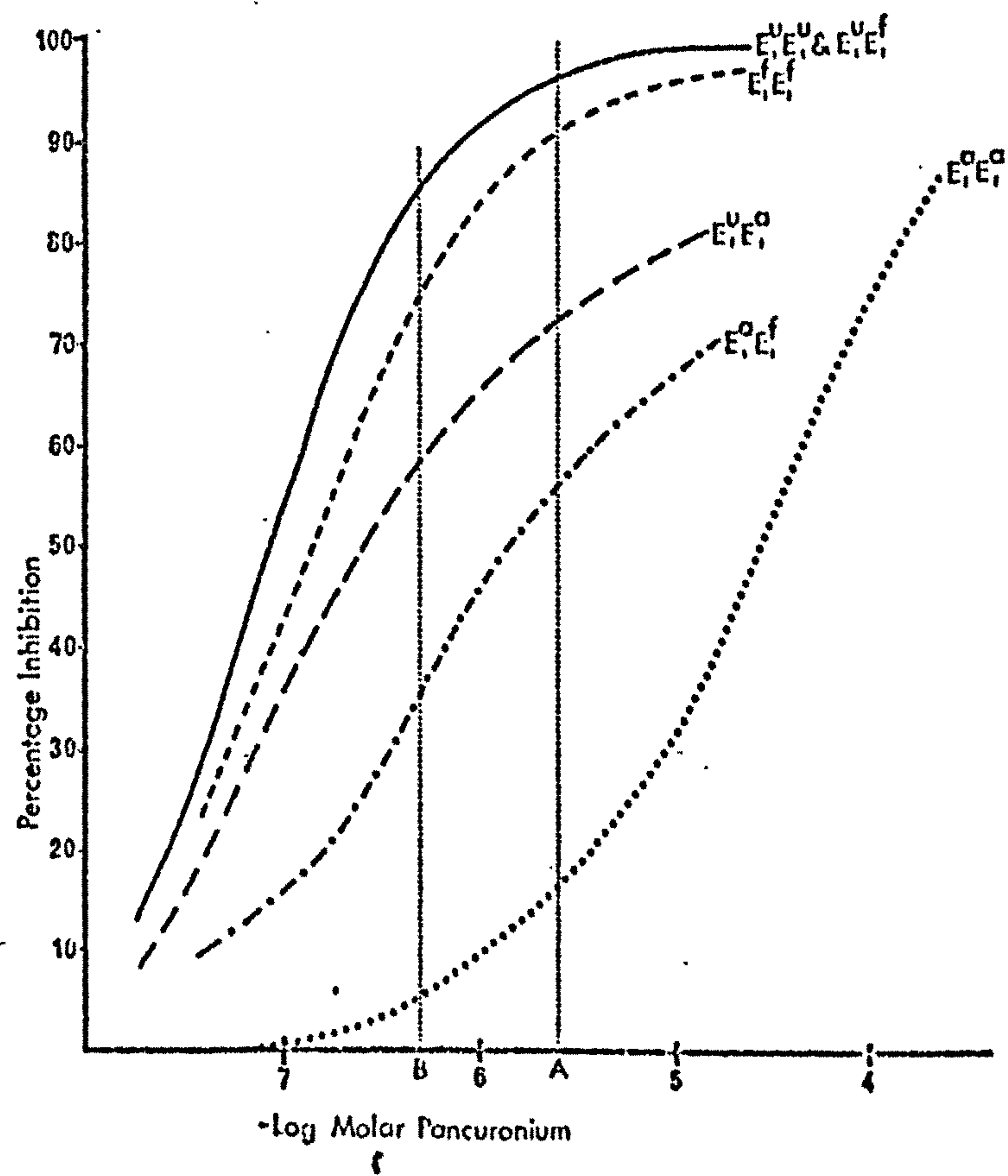


Figure 30. Inhibition of serum cholinesterase activities by pancuronium in the assay procedure of Kalow and Lindsay (1955) at 25°C. Theoretical concentrations in the blood plasma following injection of 7 mg pancuronium are shown initially at A and after 20-30 minutes at B.

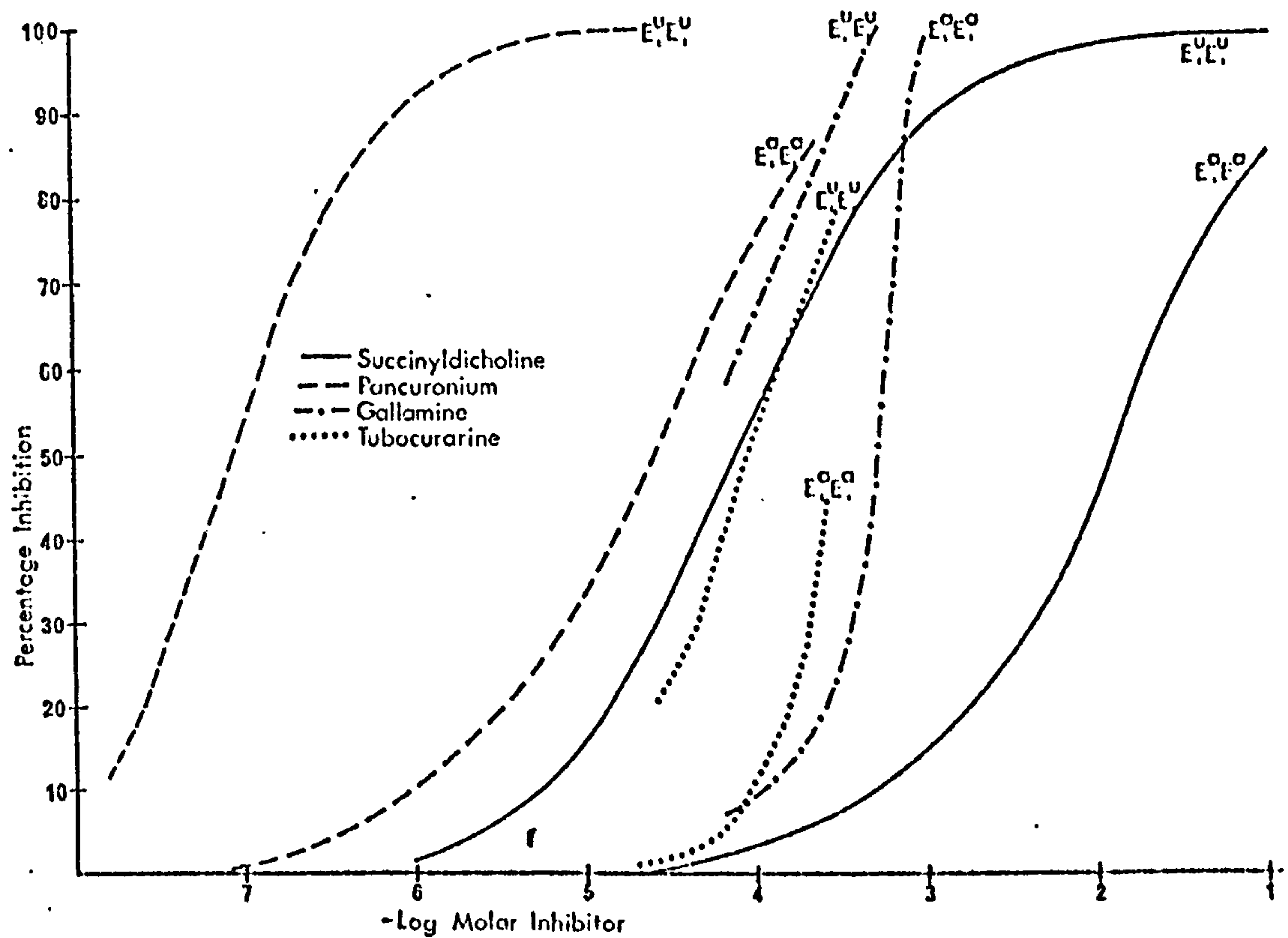


Figure 31. Comparative inhibitions of serum cholinesterase activities by some depolarising and non-depolarising muscle relaxants.

as 5% of body weight and extracellular fluid as 20-25% or 3.5 litres and 14-18 litres respectively in a 70 Kg person it can generally be assumed that 20-30 minutes after injection only 20% of the calculated initial concentration will remain in the plasma.

This highlights another difference between the inhibitions by succinylcholine and pancuronium. The maximal dose of 100 mg succinylcholine assuming immediate even distribution would give a plasma level of 100 μM and after 20-30 minutes, assuming no hydrolysis, the concentration would be 20 μM . Neither assumption of course is justified but even at these maximal possible levels of 10^{-4}M and $2 \times 10^{-5}\text{M}$ (A and B in Figure 21) barely come into the range of concentrations at which succinylcholine inhibits benzoylcholine hydrolysis in vitro. On the other hand a full dose of pancuronium of 7 mg would result in an initial plasma level of about 2.5 μM and after 20-30 minutes 0.5 μM . Both of those levels (A and B respectively, Figure 30) are well within the effective range of in vitro Pavulon inhibition of benzoylcholine hydrolysis, the initial level representing near maximum inhibition of the usual homozygote.

As far as the writer is aware this is the first recording of anticholinesterase activity in a non-depolarising muscle relaxant at in vivo concentrations. Neither gallamine nor tubocurarine exhibit inhibition at such concentration (Figure 31). A dose of 15 mg of Tubarine gives an initial plasma concentration of 5 μM and 120 mg of gallamine about 40 μM .

2. (d) Effect of Temperature on Inhibition.

Introduction

That the inhibitory action of some compounds on serum cholinesterase activity alters with temperature has been known for some time. Thus King (1965) reported that the phenotype ranges of fluoride numbers which he presented were only valid if the assays were performed in the neighbourhood of 25°C. Heilbronn (1965) also recorded an increased inhibition by fluoride with decrease in reaction temperature as did McComb, La Motta and Wetstone (1965) who found the dibucaine and succinylcholine inhibition of o-nitrophenylbutyrate hydrolysis to be less affected. In making a plea for the adoption of a standard temperature, King (1967) illustrated the variation with temperature of dibucaine and fluoride inhibition which showed that these alterations were also dependent upon the enzyme variant. These early observations are confirmed and extended in the present studies (King and Dixon, 1970; King, McQueen and Morgan, 1971).

Instrumentation, Methods and Materials

These were the same as described in the previous section except that a fourth inhibitor solution, 4 mM succinylcholine (King and Griffin, 1973) was included in some of the later experiments.

The studies on the effect of heat on the inhibition of benzoylcholine hydrolysis followed the same protocol as previously in that no allowance was made for change in optimum substrate or hydrogen ion concentrations with temperature, but the same concentrations of reactants and inhibitors were employed over the temperature range studied,

15°C to 55°C.

Serum specimens from seven usual homozygotes, three usual/fluoride-resistant heterozygotes, seven usual/atypical heterozygotes, two fluoride-resistant homozygotes, three atypical/fluoride-resistant heterozygotes and seven atypical homozygotes were studied.

Experimental Results

The variation with reaction temperature of the percentage inhibition (inhibitor number) of dibucaine, fluoride, chloride and Scoline (succinylcholine) are shown for each phenotype in Figures 32 to 37. Although in some cases, because of paucity of numbers, the mean values are barely meaningful, these were calculated and are also shown in these diagrams. The phenotype mean inhibitor numbers were collected for each inhibitor and these are illustrated in Figures 38 to 41. The ranges of inhibitor numbers established in this study for 25°C (Fig. 29) are also shown in these latter diagrams as are also the expected ranges at 37°C.

The mean variations in the four inhibitor numbers for each 5°C temperature range are listed according to cholinesterase phenotype in Tables 12 to 15.

Discussion

Although in the description of their assay procedure Kalow and Lindsay (1955) corrected activities to 26°C and reported them at this temperature, for the determination of dibucaine numbers, Kalow and Genest (1957) used a forced circulation of air to stabilise the reaction temperature

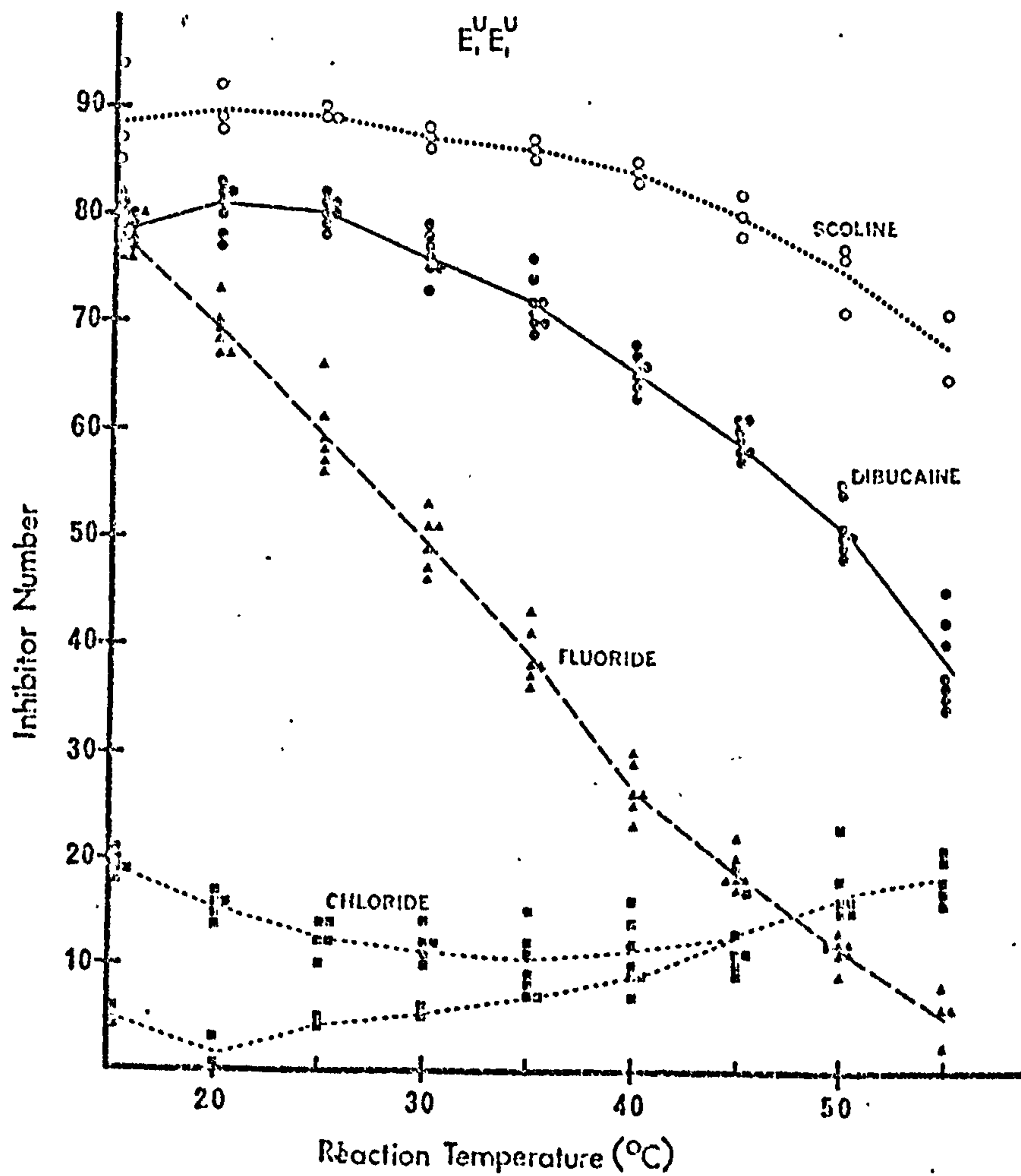


Figure 32. Variation with temperature of the inhibitor numbers of sera of cholinesterase phenotype $E_1^U E_1^U$. Each symbol represents one specimen and the lines, the mean values.

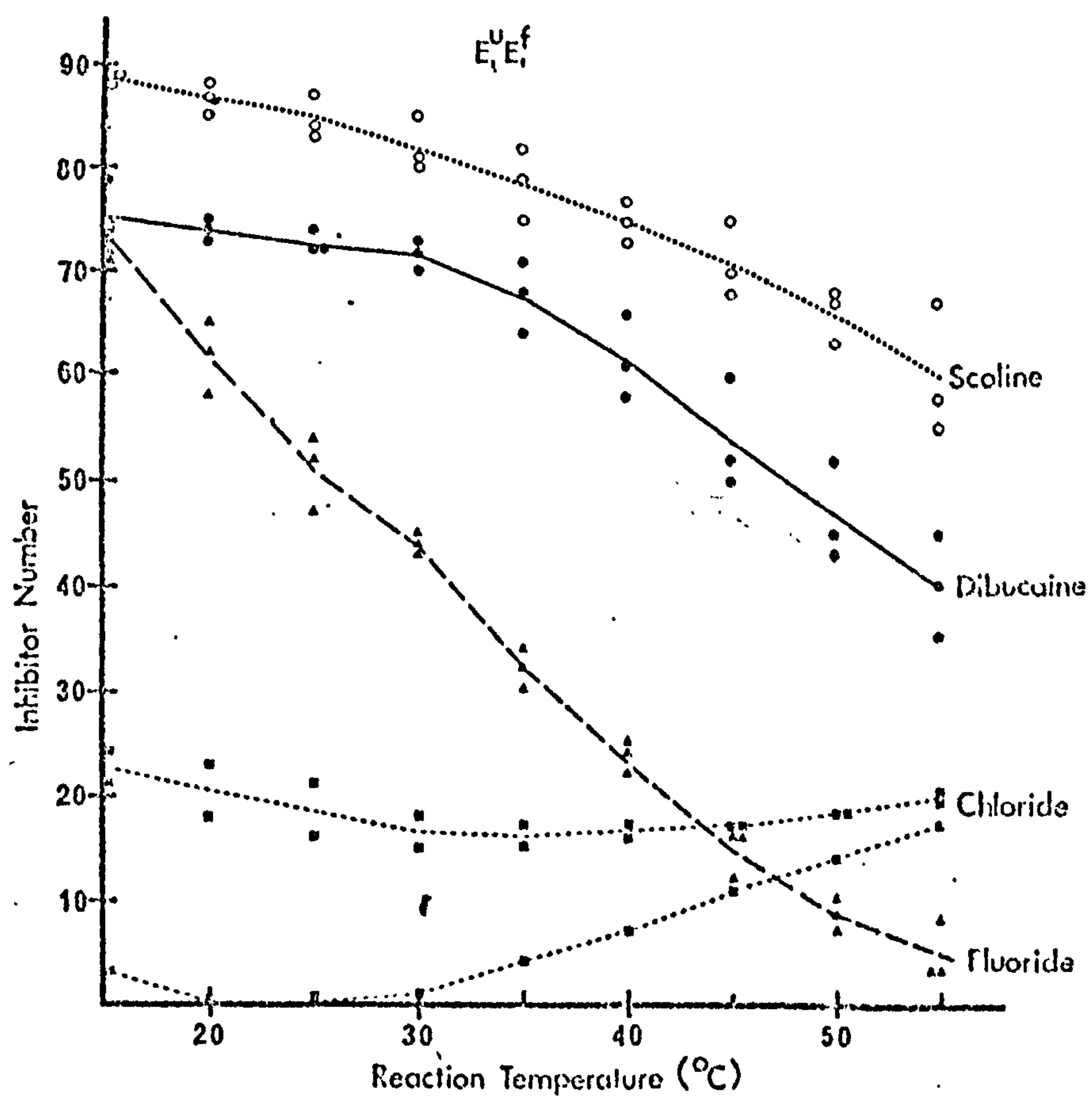


Figure 33. Variation with temperature of the inhibitor numbers of sera of cholinesterase phenotype $E_1^u E_1^f$. Each symbol represents one specimen and the lines, the mean values.

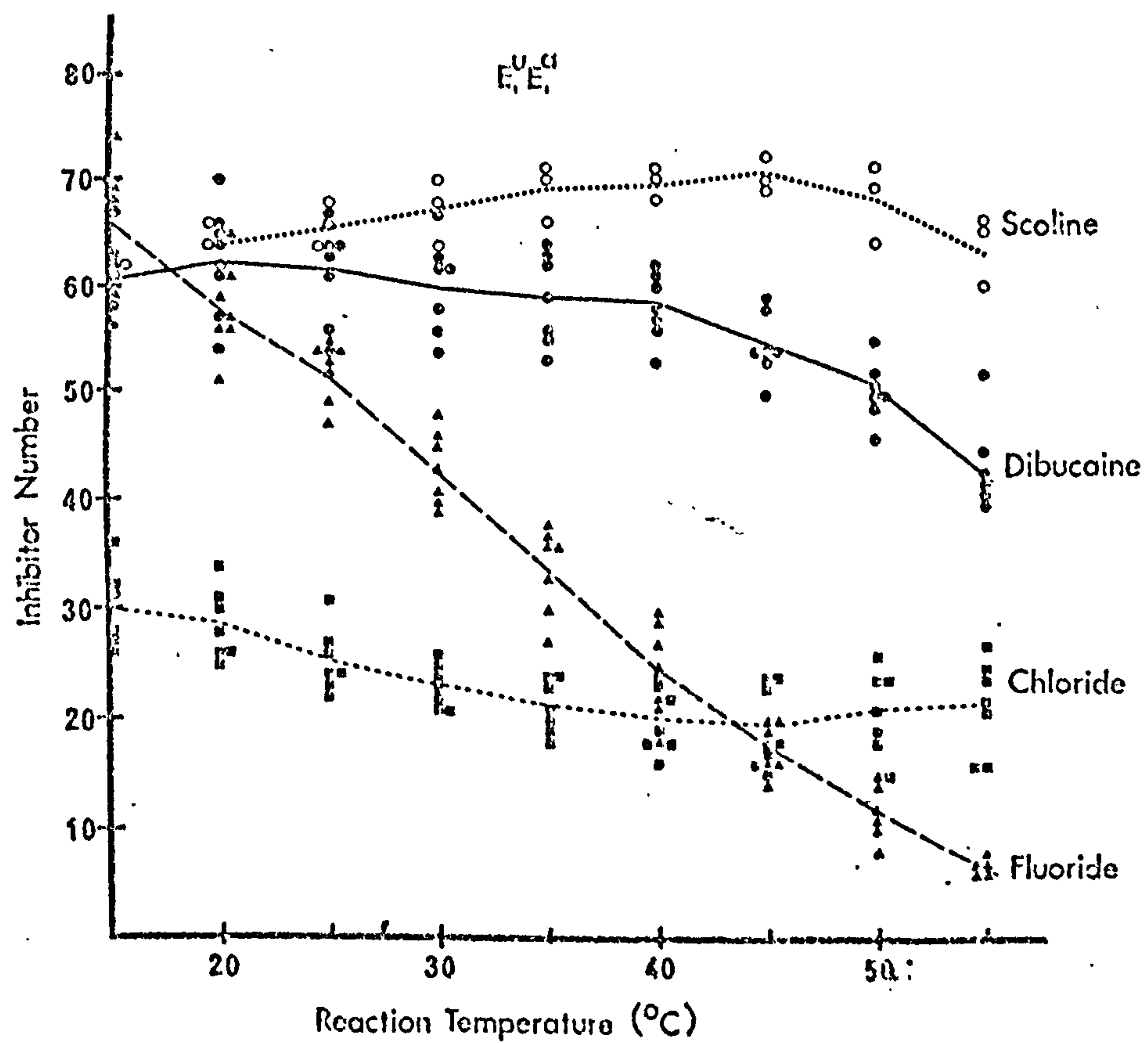


Figure 34. Variation with temperature of the inhibitor numbers of sera of cholinesterase phenotype $E_1^U E_1^A$. Each symbol represents one specimen and the lines, the mean values.

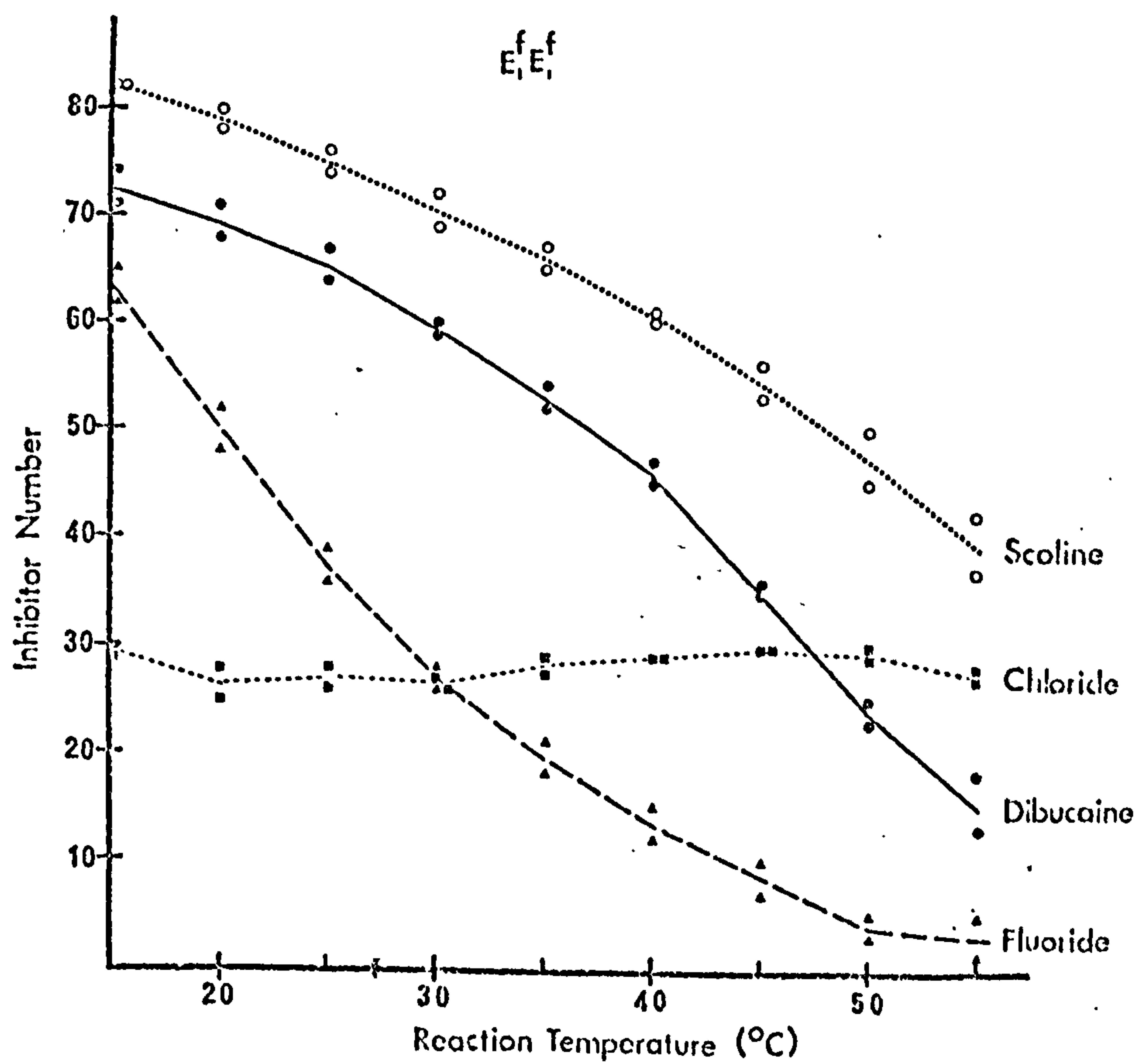


Figure 35. Variation with temperature of the inhibitor numbers of sera of cholinesterase phenotype $E_1^f E_1^f$. Each symbol represents one specimen and the lines, the mean values.

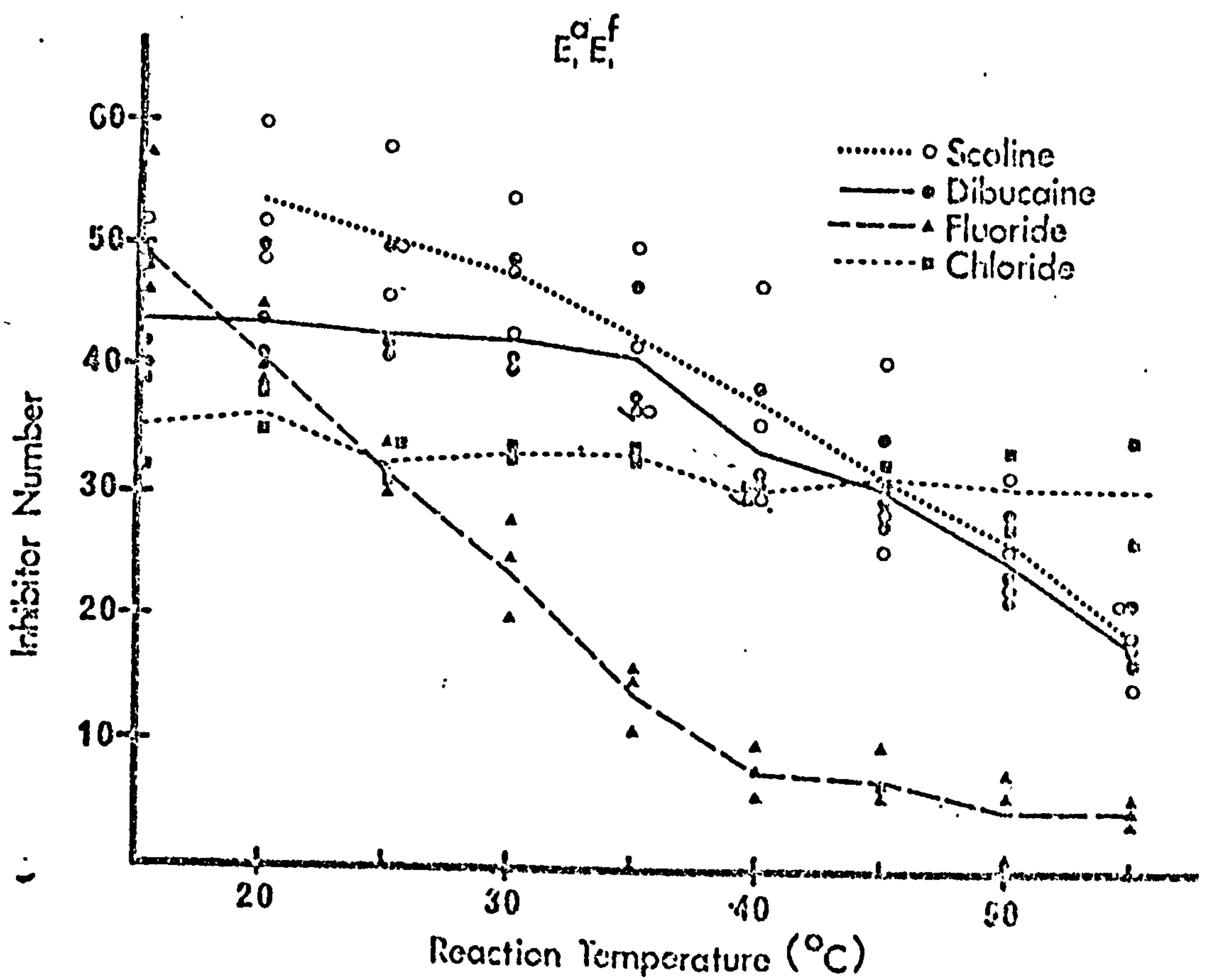


Figure 36. Variation with temperature of the inhibitor numbers of sera of cholinesterase phenotype $E_1^a E_1^f$. Each symbol represents one specimen and the lines, the mean values.

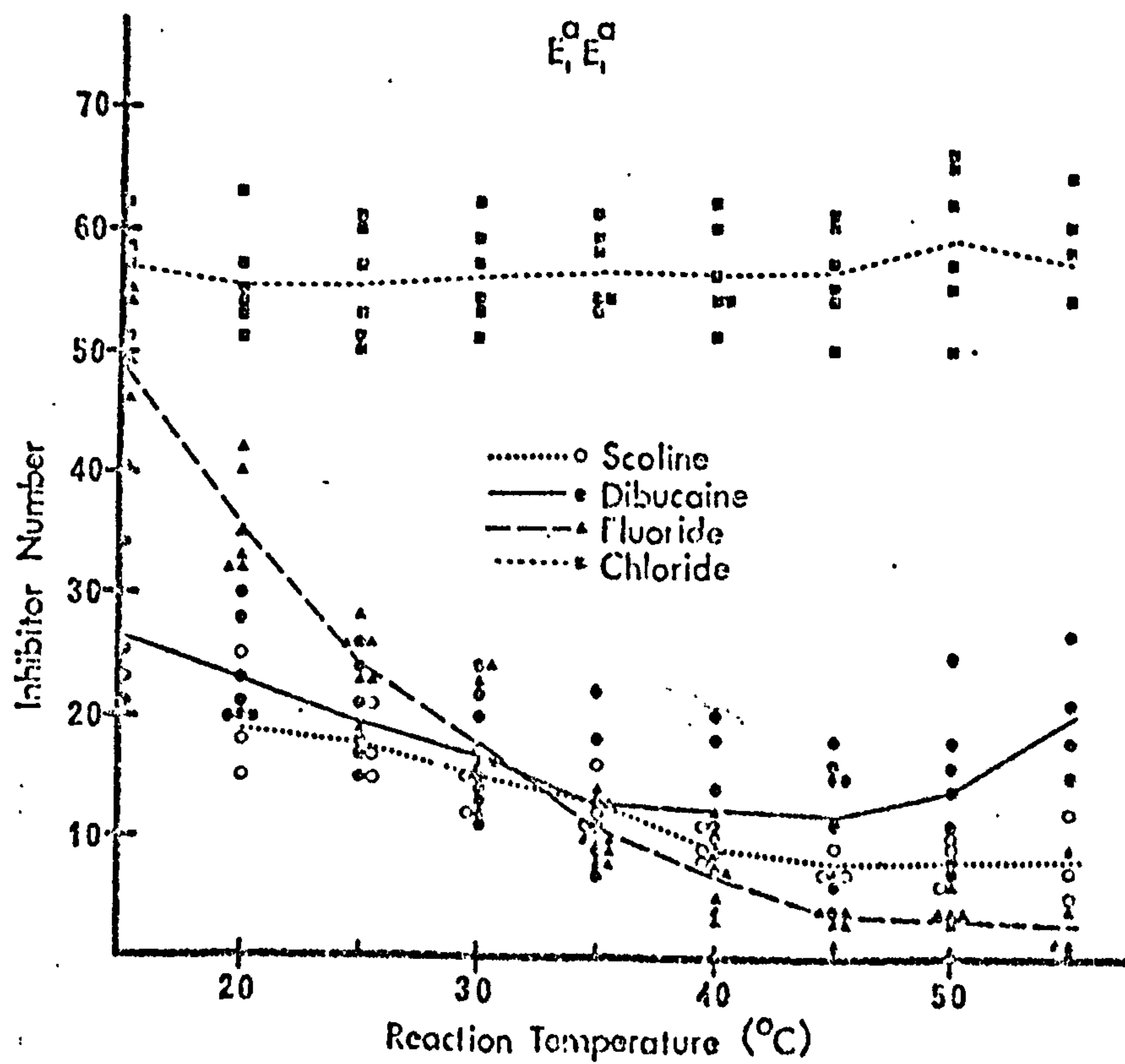


Figure 37. Variation with temperature of the inhibitor numbers of sera of cholinesterase phenotype $E_1^a E_1^a$. Each symbol represents one specimen and the lines, the mean values.

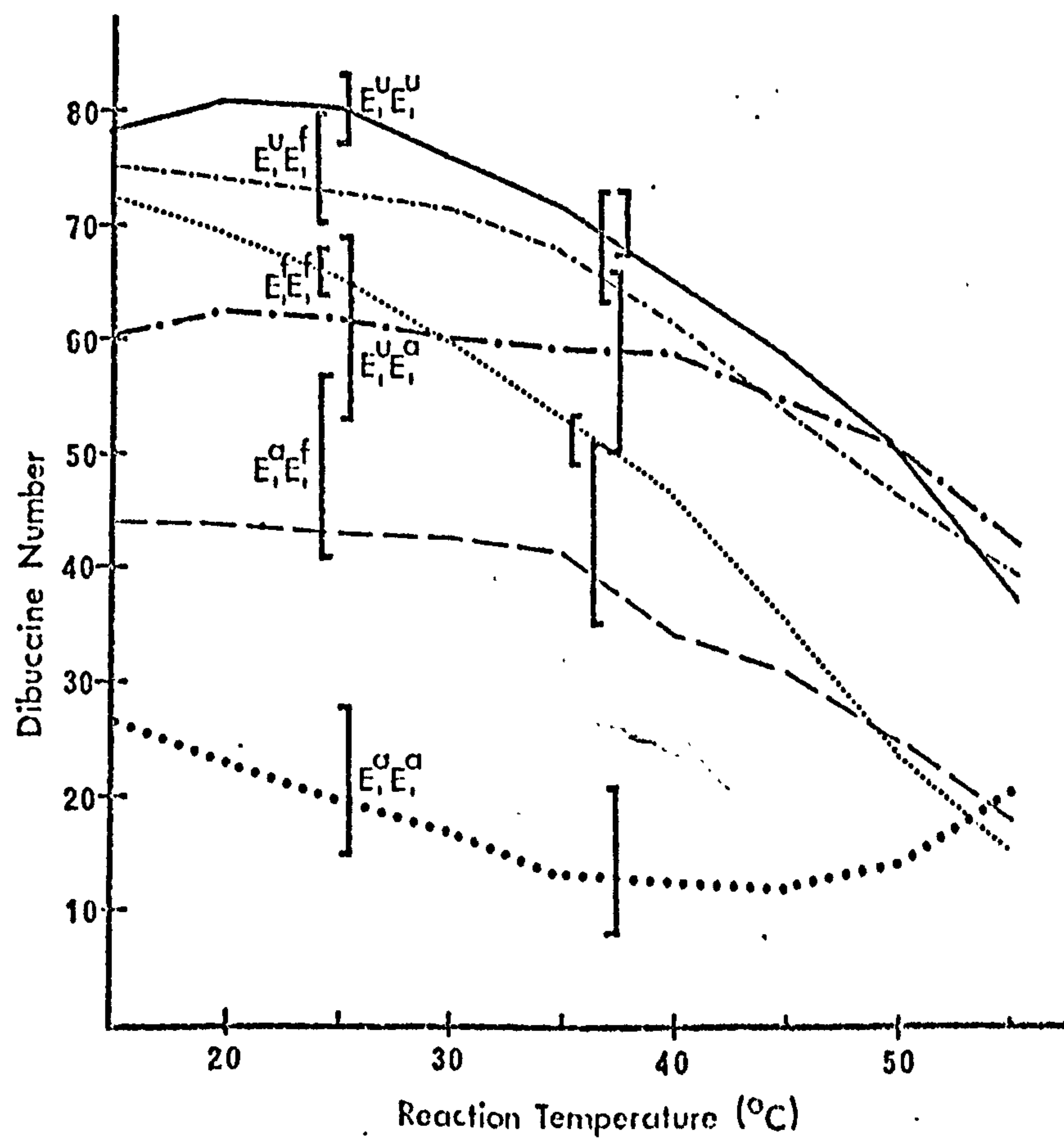


Figure 38. Variation of Dibuccaine Numbers with reaction temperature. The brackets represent the phenotype ranges encountered at 25°C and 37°C.

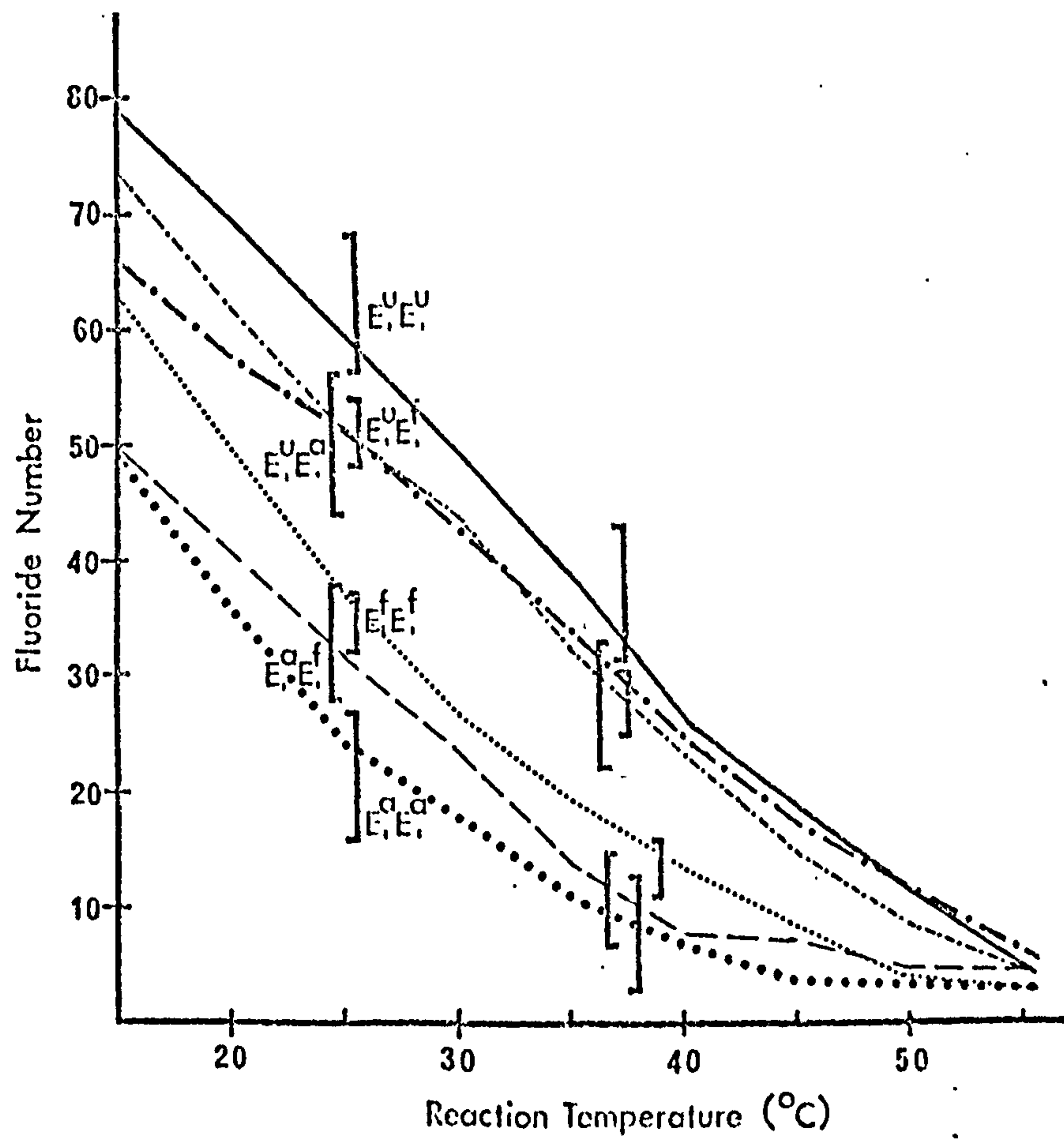


Figure 39. Variation of Fluoride Numbers with reaction temperature. The brackets represent the phenotype ranges encountered at 25°C and 37°C.

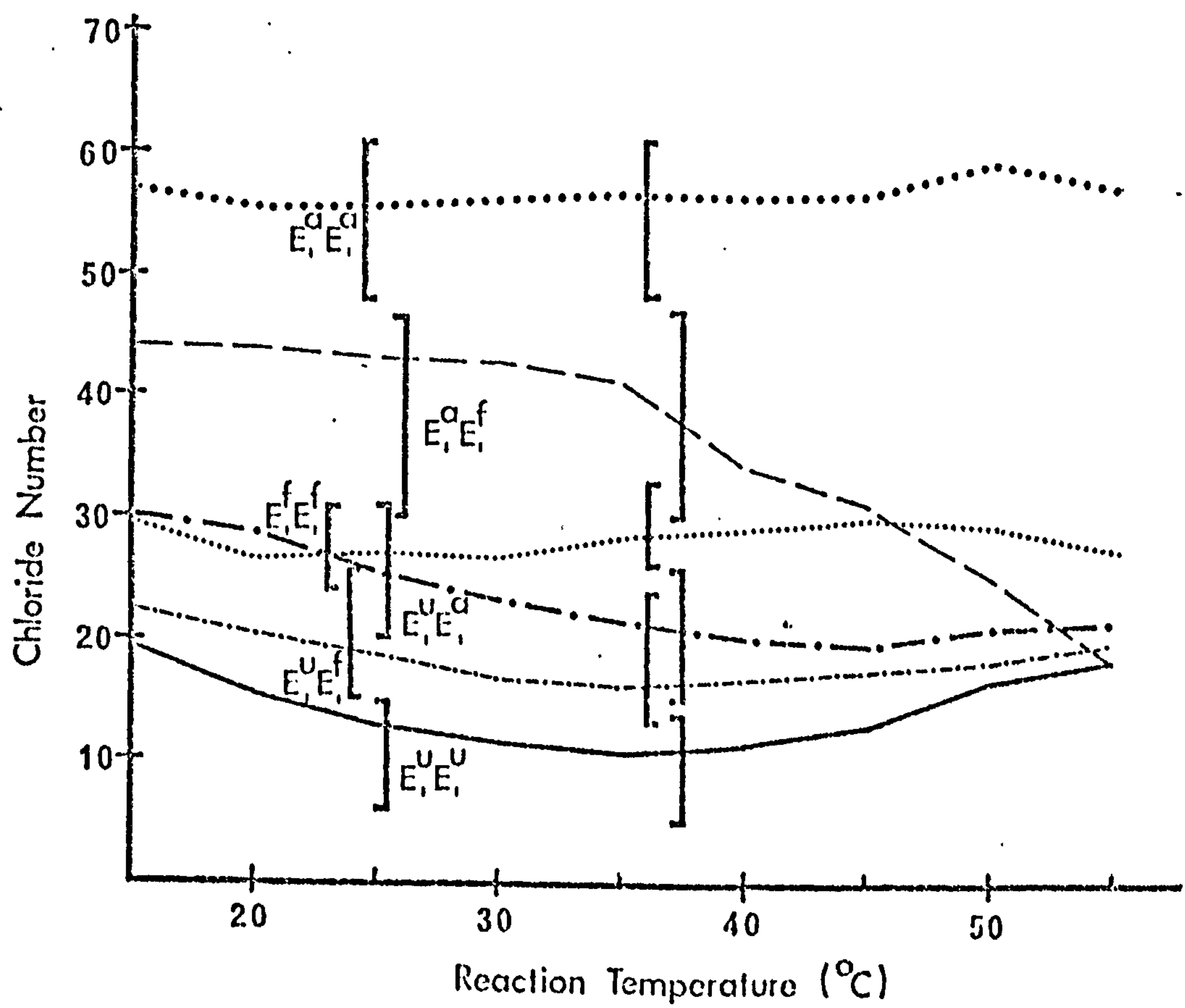


Figure 40. Variation of Chloride Numbers with reaction temperature. The brackets represent the phenotype ranges encountered at 25°C and 37°C.

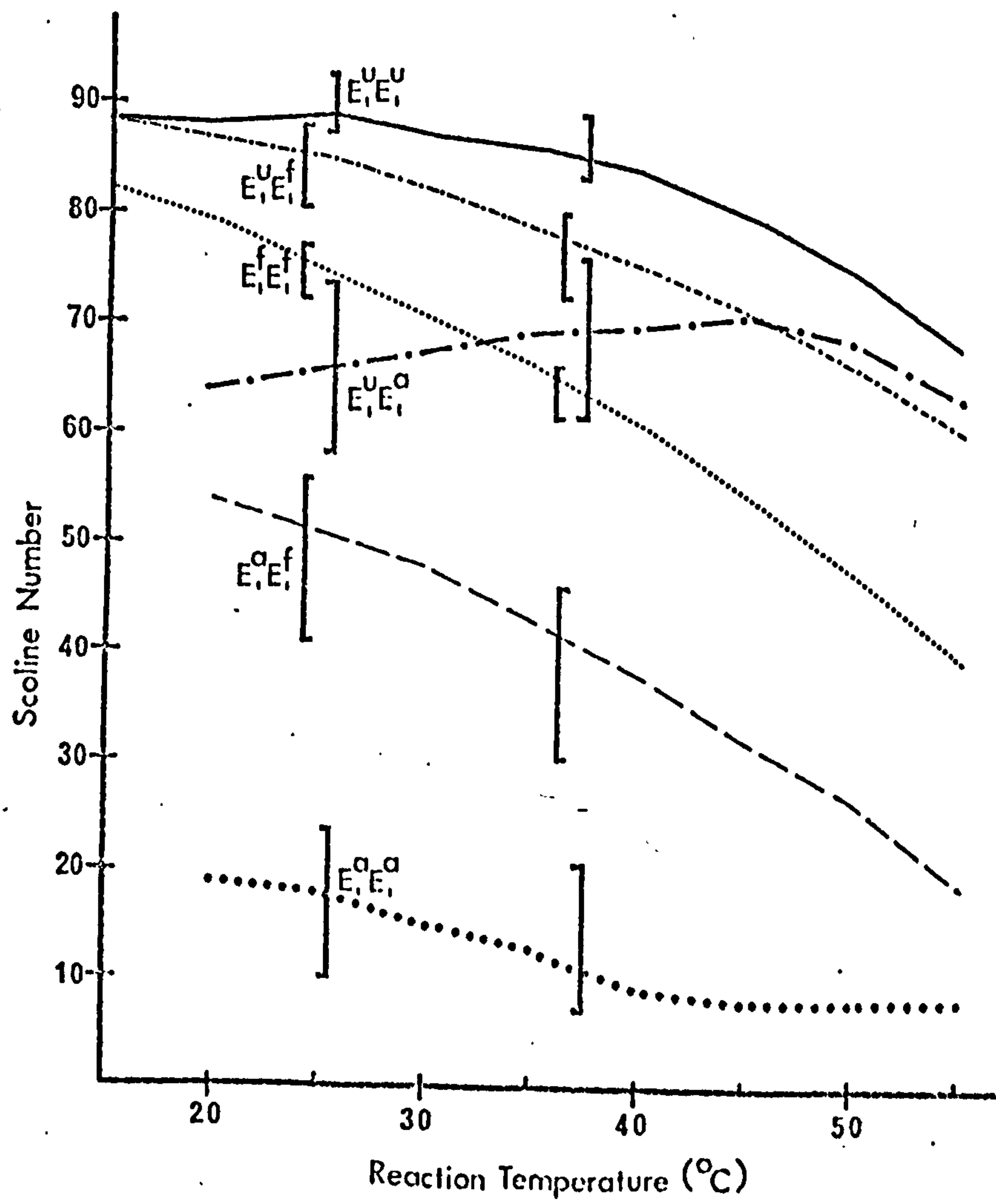


Figure 41. Variation of Scoline Numbers with reaction temperature. The brackets represent the phenotype ranges encountered at 25°C and 37°C.

between 21° and 25°. Only if there was a difference in reaction temperature between the inhibited and uninhibited assays were the reaction rates corrected to 26°C. This obviously would not correct for any variation in inhibition with temperature and the earlier figures at least for dibucaine numbers given by Kalow and co-workers are suspect on these grounds. However, Kalow and Genest did state that "the influence of temperature on the intensity of inhibition was negligible within the narrow range from 21-25°C", indicating that they were aware of the phenomenon. On the other hand, Harris and Whittaker (1961) in introducing the fluoride number did not mention any reaction temperature merely stating that it was determined "in exactly the same way as dibucaine number determination". It was assumed therefore by some workers (Lee and Robinson, 1967) that the phenotype ranges of fluoride number were determined at 21°-25°C, as they well may have been although Whittaker (1964, 1967) did stipulate a reaction temperature of 26.5°C. This was also the temperature at which she determined chloride numbers (Whittaker, 1968a,b) while King and Griffin (1973) estimated succinylcholine inhibition at 25°C.

In general, in the temperature range studied, all inhibitions were highest at the lower temperatures, except for the anomalous chloride inhibition of the family reported by King and Dixon (1970) as having low or zero chloride numbers at 25°C. These individuals were classified as either usual homozygotes or usual/fluoride-resistant heterozygotes and the loci of chloride numbers are shown in Figures 32 and 33 respectively. It was noted in these

inhibition experiments, as in the previous benzoylcholine hydrolysis studies, that the presence of a silent gene did not alter phenotype behaviour.

Chloride inhibition was the least affected by change in reaction temperature and showed a similar pattern for all variants. As the temperature increases there is a small decrease in inhibition reaching minima about 35°C for the usual enzyme, 20°C - 30°C for the fluoride-resistant variant and 40°C - 45°C for the atypical isoenzyme after which the inhibitor numbers increase. It is interesting to note that the heterozygous sera demonstrate intermediate minima and although it would be foolhardy to draw any conclusions from two experiments, the erratic behaviour of the chloride number with the atypical/fluoride-resistant sera could possibly be construed as mirroring the widely-divergent minima of the two constituent variants (Figure 40). Variation with temperature of chloride inhibition of cholinesterase activity does not appear to be recorded in the literature.

For all phenotypes, dibucaine and succinylcholine inhibitions follow fairly similar patterns (Figures 32 to 37). There is, in general, except for the fluoride-resistant enzyme a tendency to show a maximum about 20°C , followed by a moderate decline in number to about 35°C - 40°C and then, except for the atypical variant which shows an increase, a more rapid decrease in inhibition. It is difficult to explain the anomalous behaviour of the succinylcholine inhibition of the usual/atypical heterozygous sera except that these three specimens came from siblings and may not

be representative of the general atypical heterozygote population.

Between 25°C and 37°C the dibucaine number falls by 11 in the usual homozygote, by 15 for phenotype $E_1^f E_1^f$ and by 7 for the atypical homozygote with generally intermediate values for heterozygotes (Figure 24 and Table 10). Between 20°C and 40°C the average dibucaine inhibition of the usual enzyme decreases from 81% to 66% or a fall of 0.75 per degree in the dibucaine number. Over the same temperature range, Lee and Robinson (1967) reported a reasonably similar figure of 0.6 for what they termed the $\frac{\Delta DN}{\Delta t}$. Except for this study (King and Dixon, 1970) the literature does not appear to record any information regarding the variation with temperature of dibucaine inhibition of cholinesterase activity for any of the other genetic variants.

Over the range 25°C to 37°C the mean scoline number decreases by 4 for the usual homozygote, by 11 for the fluoride resistant homozygote and by 6 for the atypical homozygote, values which are less than, but follow a similar trend to, the dibucaine number (Figure 41 and Table 15). For the usual homozygote the change in scoline number is less than 0.3 per degree between 20°C and 40°C.

The picture for fluoride inhibition differs from the others, qualitatively in that there are no maxima or minima and, quantitatively in that there are greater changes amounting to reductions in the mean fluoride number of 25, 21 and 15 for the phenotypes $E_1^u E_1^u$, $E_1^f E_1^f$ and $E_1^a E_1^a$ respectively between 25°C and 37°C. The heterozygotes show the appropriate intermediate changes (Figure 39 and Table 13).

For the usual homozygote over the temperature range 20° - 40° C there is a change of 2.2 in the fluoride number per degree. This is the identical value given by Lee and Robinson (1967) but is less than the 3.1 found by McComb, La Motta and Wetstone (1965). The latter authors employed o-nitrophenylbutyrate as substrate however and the values are therefore not truly comparable.

The values given in the preceding paragraphs, particularly those referring to the fluoride number, tend to give the superficial impression that the inhibition of the atypical enzyme is least affected by temperature change. This results from the fact that, except for chloride, the inhibition of the atypical enzyme is much lower than the other variants at 20° - 25° C. Perhaps the most valid index of sensitivity by phenotype is to take the inhibition at 25° C as standard and record the increase in temperature necessary to reduce the inhibition by half. Expressed in this manner, fluoride inhibition is reduced to 50% at 34° C, 35° C and 39° C for the phenotypes $E_1^a E_1^a$, $E_1^f E_1^f$ and $E_1^u E_1^u$ respectively (Figure 39). By the same procedure the dibucaine number at 25° C of the fluoride-resistant homozygote is reduced by half at 46° C but this does not occur with any other phenotype at less than 50° C (Figure 38).

Harris, Lehmann and Silk (1960) reported that there was "significant correlation between the dibucaine numbers and cholinesterase levels within the 'intermediate' group", that is the atypical heterozygote group, and indeed there is in general a correlation between the dibucaine number and the cholinesterase activity between the groups. The

phenotypes with lowest dibucaine numbers tend to have lowest serum activities (Figures 14 and 15) and there is a distinct correlation between dibucaine number and suxamethonium sensitivity (Figures 2 and 29) with duration of apnoea under standardised conditions. This general precept only fails in the case of the fluoride-resistant homozygote with which dibucaine inhibition is similar to that of the atypical heterozygote, although the $E_1^f E_1^f$ phenotype is invariably sensitive but $E_1^u E_1^a$ individuals are rarely and unpredictably so. Again however this anomaly is resolved by a study of Figure 38 where it is seen that the dibucaine inhibition of the fluoride-resistant homozygote is more severely affected by increase in temperature than any other phenotype and at 37°C the dibucaine number is sensibly that of the $E_1^a E_1^f$ genotype which is invariably suxamethonium sensitive. The same applies to the other inhibitors, fluoride (Figure 39), chloride (Figure 40) and scoline (Figure 41) and Tables 12-15.

Once more therefore this study has indicated the inadequacy of measurements at lower reaction temperatures to predict that which will happen at the functional temperature of the enzyme, 37°C , and for a true comparability between inhibitor number and sensitivity to suxamethonium the inhibition assays should be performed at 37°C .

In the absence of a thermostatically controlled spectrophotometer it is the ambient temperature which dictates the reaction temperature of cholinesterase assays. Schneider and Willis (1958) and Henry, Chiamori, Golub and Berkman (1960) found the temperature in the cell compartment

Table 12 Variation of Percentage Inhibition with Temperature

DIBUCAINE NUMBER						
Temperature Range (°C)	$E_1^u E_1^u$	$E_1^u E_1^f$	$E_1^u E_1^a$	$E_1^f E_1^f$	$E_1^a E_1^f$	$E_1^a E_1^a$
15 - 20	+2.5	-1.5	+1.5	-3.0	0	-3.5
20 - 25	-1.0	-1.5	-0.5	-4.0	-0.5	-3.5
25 - 30	-4.0	-1.0	-2.0	-6.0	-0.5	-3.0
30 - 35	-4.0	-4.0	-1.0	16.5	-2.0	-3.5
35 - 40	-6.5	-6.0	-0.5	-7.0	-7.0	-0.5
40 - 45	-7.0	-7.5	-4.0	-9.5	-3.0	-0.5
45 - 50	-7.0	-7.5	-3.5	-11.5	-6.0	+2.0
50 - 55	-12.5	-6.5	-8.0	-8.5	-7.0	+6.0

Table 13 Variation of Percentage Inhibition with Temperature

FLUORIDE NUMBER

Temperature Range (°C)	$E_1^u E_1^u$	$E_1^u E_1^f$	$E_1^u E_1^a$	$E_1^f E_1^f$	$E_1^a E_1^f$	$E_1^a E_1^a$
15 - 20	-9.0	-11.5	-8.5	-13.5	-9.0	-13.0
20 - 25	-10.5	-10.5	-6.0	-12.5	-9.0	-11.5
25 - 30	-10.0	-7.0	-9.0	-10.5	-7.5	-6.5
30 - 35	-10.5	-12.0	-9.0	-7.5	-10.0	-7.0
35 - 40	-12.5	-9.0	-9.5	-6.0	-6.0	-4.0
40 - 45	-8.0	-8.5	-7.0	-5.0	-0.5	-3.0
45 - 50	-7.0	-6.0	-6.0	-4.5	-2.5	-0.5
50 - 55	-6.5	-4.0	-5.5	-1.0	0	-0.5

Table 14 Variation of Percentage Inhibition with Temperature

CHLORIDE NUMBER

Temperature Range (°C)	$E_1^u E_1^u$	$E_1^u E_1^f$	$E_1^u E_1^a$	$E_1^f E_1^f$	$E_1^a E_1^f$	$E_1^a E_1^a$
15 - 20	-4.0	-2.0	-1.0	-3.0	+1.1	-1.5
20 - 25	-3.0	-2.0	-3.5	+0.5	-4.0	0
25 - 30	-1.5	-2.0	-2.5	-0.5	+1.0	+0.5
30 - 35	-0.5	-0.5	-1.5	+2.0	0	+0.5
35 - 40	+1.0	+0.5	-1.5	+1.0	-3.0	-0.5
40 - 45	+1.5	+0.5	-0.5	+1.0	+1.5	+0.5
45 - 50	+3.0	+1.0	+1.5	-0.5	-1.0	+2.5
50 - 55	+2.0	+1.5	+0.5	-2.0	0	-2.0

Table 15 Variation of Percentage Inhibition with Temperature

SCOLINE NUMBER

Temperature Range (°C)	$E_1^u E_1^u$	$E_1^u E_1^f$	$E_1^u E_1^a$	$E_1^f E_1^f$	$E_1^a E_1^f$	$E_1^a E_1^a$
15 - 20	-0.5	-2.0	-	-3.0	-	-1.5
20 - 25	+1.0	-1.5	+2.0	-4.0	-2.5	0
25 - 30	-2.0	-3.0	+1.5	-4.5	-3.0	+0.5
30 - 35	-1.0	-3.5	+1.5	-5.0	-5.0	+0.5
35 - 40	-2.5	-3.5	+0.5	-5.5	-5.5	0
40 - 45	-3.5	-4.0	+1.0	-6.0	-5.5	0
45 - 50	-5.0	-5.0	-2.5	-7.0	-5.5	+2.5
50 - 55	-6.5	-6.0	-5.0	-8.5	-7.5	-2.0

of their spectrophotometers to be 5° to 7°C above the ambient room temperature. In these laboratories in Glasgow Royal Infirmary, the bench temperature, out of direct sunlight, has been found to fluctuate between 18°C and 32°C during the year. This is probably a general range in these islands but is probably wider elsewhere. It was doubtless an appreciation of such high ambient temperatures which prompted the International Union of Biochemistry (1965) to reject the earlier advocacy of 25°C by its Commission on Enzymes (I.U.B. 1961) and to recommend that 30°C be used as the standard temperature. In many parts of the world however, even in these northern latitudes in summer-time, to maintain a cuvette temperature of 30°C requires thermostatic cooling of the instrument. This difficulty, the fact that a valid index of some enzymes is only obtained by assay at their physiological temperature (King and Dixon, 1969; King and Morgan, 1970) and the desirability of exploiting the increased activity, and hence better precision, at higher temperatures in the automation of enzyme assays, compellingly suggest the universal adoption of 37°C as the standard temperature for enzyme assays at least in clinical biochemistry (King, 1972).

The variation with temperature in the inhibition of the allelic serum cholinesterase isoenzymes would appear to be a counter-argument to this proposal. This only applies to fluoride however under the assay conditions employed in this study since Figures 38, 40 and 41 show that dibucaine, chloride and succinylcholine respectively can still provide a means of differentiating the cholinesterase phenotypes at 37°C . With fluoride on the other

hand (Figure 39) the inhibition is extremely sensitive to temperature change so that even at 30°C, far less 37°C it cannot provide a reliable means of distinguishing the cholinesterase variants.

As it is, even with current practices, the revelation that between 18°C and 32°C, the ambient temperature range in these laboratories, the fluoride number alters by 1.4 to 2.2 per degree change in reaction temperature, emphasises the need for strict temperature control of the reaction. From Figure 39 it can readily be seen that an alteration in reaction temperature of 2°C-4°C suffices to change the fluoride number from one phenotype range to that of the next. It has been recorded (King, McQueen and Morgan, 1971) that if the assays were performed at 20°C, the fluoride-resistant homozygote would give values for inhibitor numbers except scoline, which could lead to a classification of $E_1^u E_1^a$ or even $E_1^u E_1^f$. It was even speculated that the rarity of the phenotype $E_1^f E_1^f$ (Lehmann and Liddell, 1969) might in part be due to a general failure to appreciate the necessity for strict temperature control.

Kalow and Davies (1958) postulated that the structural difference between the usual and atypical enzymes involved an alteration in the so-called "anionic site" at which the positively charged grouping of some substrates and inhibitors (Figures 1 and 20) finds attachment. To check this hypothesis Bamford and Harris (1964) used the 'neutral' substrate α -naphthyl acetate with which it was argued that the anionic state would play little part while the postulated esteratic site would be the same for both enzymes. These later

workers found that with the positively-charged inhibitors, dibucaine and RO2-0683 (Kalow and Davies, 1958), at appropriate concentrations, differential inhibitions were obtained with α -naphthyl acetate similar to those with benzoylcholine. However they found the differential inhibition of the enzymes with fluoride was minimal with α -naphthyl acetate in contrast to that obtained with a benzoylcholine substrate and concluded

"However, some difference in the degree of inhibition of the two enzymes by fluoride does appear to be present even when naphthyl acetate is used as substrate, and when therefore neither substrate nor inhibitor is positively charged. This observation is not readily explained by the hypothesis in its simplest form, and it will clearly be difficult to assess its significance until more is known about the mode of action of the F^- ion on the enzyme".

Bamford and Harris (1964) followed the hydrolysis of the α -naphthyl acetate substrate at $37^{\circ}C$ and the reduction in the observed differential inhibition by fluoride was probably entirely due to the reaction temperature and little influenced by the substrate.

3. Thiocholine Ester Hydrolysis.

Thiocholine Ester Hydrolysis

Koelle (1957) used acetylthiocholine as a substrate for histochemical localisation of cholinesterase. The sulphur analogues of aliphatic choline esters have also been widely used in cholinesterase assays employing various indicator reactions to follow the liberation of thiocholine (Page 8). The most successful of these has been the reaction (Ellman, 1959) with 5,5'-dithiobis-(2-nitrobenzoic) in which the liberation of the yellow anion of 5-thio-2-nitrobenzoic acid (Figure 42) is used to follow the enzymic hydrolysis at wavelengths 405-420 nm. Ellman, Courtney, Andres and Featherstone (1961) were the first to employ this principle for assay of acetylcholinesterase and Garry and Routh (1965) for human serum cholinesterase. Since then numerous modifications (Augustinsson, 1971) have appeared, all employing acetylthiocholine or butyrylthiocholine as substrate, except for the recently published Selected Method of the American Association of Clinical Chemists which uses propionylthiocholine (Dietz, Rubinstein and Lubrano, 1973).

The validity of using thiocholine esters as substrates has been established therefore and the kinetics of the reactions apparently well investigated, certainly for the acetyl and butyryl esters. Augustinsson (1971) concluded that "the principle described by Ellman and his co-workers represents one of the most straightforward methods developed for cholinesterase experiments". Only one disadvantage can be attributed to the principle. Unlike benzoylcholine for which Kalow, Genest and Staron (1956) estimated a half-life at bench temperature of 2½ years, the thiocholine ester

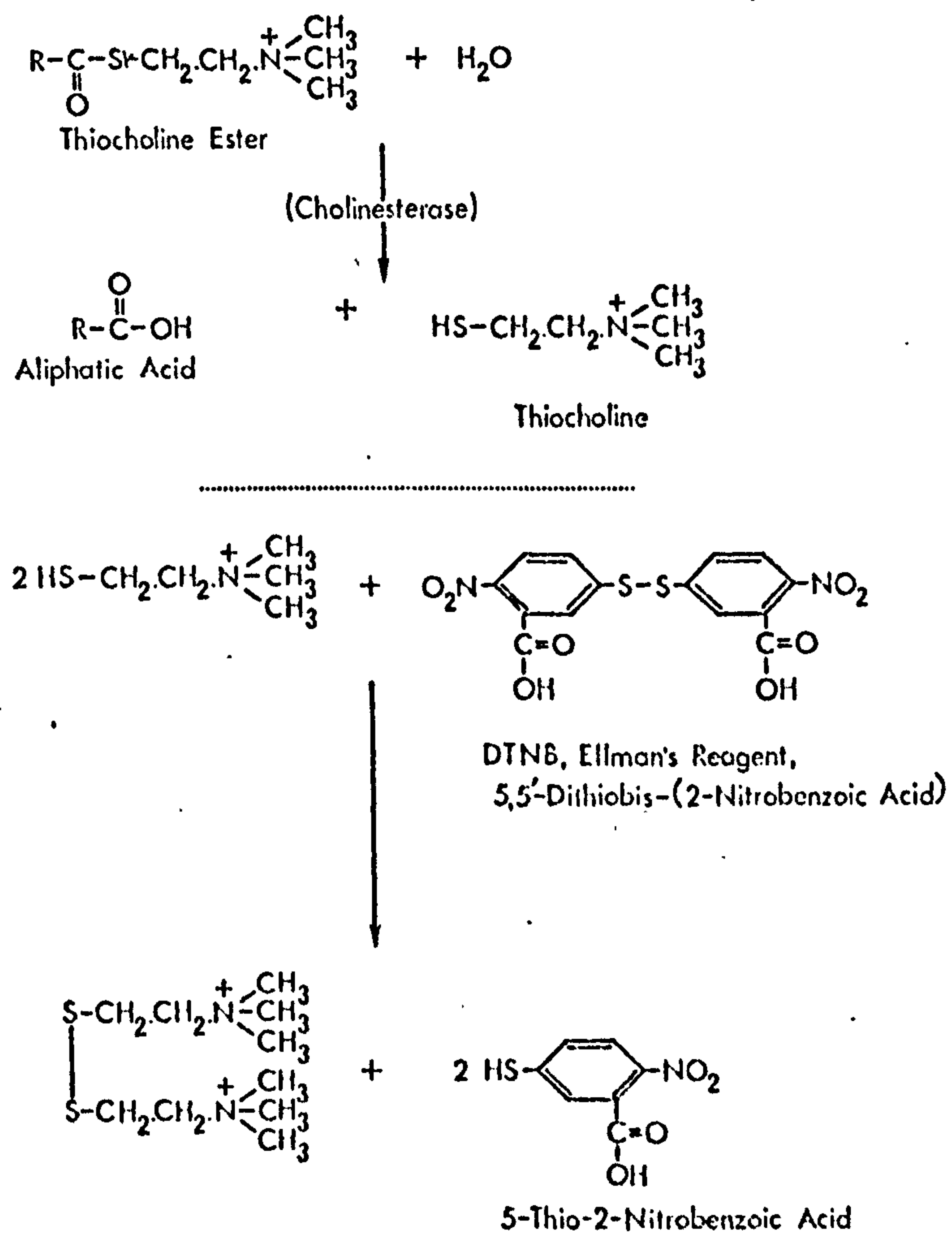


Figure 42. The reactions involved in estimating thiocholine ester hydrolysis.

solutions are not very stable and non-enzymic hydrolysis cannot be ignored.

Initially it had been thought that succinylcholine would be an excellent substrate for differentiating the succinylcholine sensitive individuals by cholinesterase activity alone. However preliminary experiments in following the change in pH by means of the Astrup micro-equipment (Johnson and Whitehead, 1965) revealed this to be too insensitive. This did lead to the studies of succinylcholine as a differential inhibitor however (p. 80 et seq.) and the search for a commercial source of succinylthiocholine. This substrate has only recently become commercially available (Boehringer Corporation) following the studies of Gibson and Brown (1972).

Instrumentation, Methods and Materials

Initially, studies with thiocholine esters were carried out at 410 nm on a Unicam SP800 recording spectrophotometer with scale expansion to a Servoscribe recorder and temperature control given by a Shandon circulating heater and waterbath. The major part of the work herein reported however was carried out on an LKB 8600 Reaction Rate Analyzer with 410 nm filter system. This is a fixed-temperature instrument, operating at 37°C and therefore not capable of accommodating temperature studies. It was modified however by Mr. J. Gilchrist, the LKB Scottish Region Engineer to have a variable temperature control (Figure 43) between 20°C and 50°C in steps of 5°C (King and Ritchie, 1974). At first, for temperatures below ambient, this instrument was operated in the cold room, but was more conveniently employed in con-



Figure 43. Temperature modification to the LKB 8600 Reaction Rate Analyzer.



Figure 44. Complete instrumentation for reaction rate analysis of thiocholine ester hydrolysis comprising modified LKB 8600 analyzer, LKB 8610 cooling stage, LKB 6500 flat bed recorder, LKB 8200 calculator and Data Dynamics 390 ASR teletype.

junction with an LKB 8610 Cooling Stage for which the generous support of the New Medical Developments Committee of the Scottish Home and Health Department is gratefully acknowledged.

This last apparatus holds the reaction temperature at 25°C against an ambient temperature of 32.5°C , the highest experienced during its period of operation. As a cooling device it is very slow however, taking 2 hours to cool the reaction tunnel of the analyzer from 45°C to 25°C at ambient temperatures around 30°C . It was found that keeping the cuvette racks in the deep freeze (-20°C) and processing these through the tunnel reduced the temperature in a matter of minutes. The same result was obtained more conveniently by a solid brass block of identical dimensions to the cuvette rack which was manufactured to order by Watson-Balgray Engineering of Glasgow. Latterly, the instrumentation was largely automated by the addition of an LKB 8200 Calculator and Data Dynamics 390 ASR teletype (Figure 44). The LKB 8600 Reaction Rate Analyzer was further modified in that the timing-cam was cut for a 30-second differential. For the experiments described it was found that a 30-second scan was sufficient for precise, accurate results and the reduced pre-incubation time of $7\frac{1}{2}$ minutes was just sufficient for the rack, cuvette and contents to reach the selected reaction temperature (King, 1973). Initially temperature control was checked by means of a Wayne Kerr contact thermometer and latterly by a Light Laboratories 3GID Temperature Monitor with an IRM probe.

In addition to the reagents previously employed (p. 43 and p. 82) acetylthiocholine iodide was obtained from

Boehringer Corporation (London) and BDH Chemicals, propionylthiocholine iodide from Sigma Chemical Corporation, butyrylthiocholine iodide from BDH Chemicals, succinyldithiocholine iodide from Boehringer Corporation (London) and Ellman's reagent, 5,5'-dithio-bis(2-nitrobenzoic acid) from Boehringer Corporation (London) and BDH Chemicals. No significant difference was found between reagents from alternative sources.

For these studies the following solutions were prepared:

- (i) 50 mg DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) dissolved in 50 ml 67 mM Sørensen phosphate buffer, pH 7.4.
- (ii) 160 mg acetylthiocholine iodide dissolved in 10 ml of reagent (i).
- (iii) 165 mg propionylthiocholine iodide dissolved in 10 ml of reagent (i).
- (iv) 105 mg butyrylthiocholine iodide dissolved in 10 ml of reagent (i).
- (v) 60 mg succinyldithiocholine iodide dissolved in 10 ml of reagent (i).

Addition of 100 μ l of these substrate-DTNB solutions to give a reaction volume of 1.1 ml produced final substrate concentrations of 5 mM acetylthiocholine, 5 mM propionylthiocholine, 3 mM butyrylthiocholine and 1 mM succinyldithiocholine.

The enzyme sources for these studies were human sera, the phenotypes of which had been determined as detailed in the preceding sections. For assay of cholinesterase activity on the LKB 8600, 20 μ l of the required serum was diluted with 20 ml of 67 mM phosphate buffer, pH 7.4. One millilitre

of this diluted serum was then placed in a cuvette and the reaction started by the automatic priming with 100 μ l of substrate-DTNB solution. For the inhibition studies, 20 μ l of serum was diluted with 10 ml of buffer; 0.5 ml of this dilution and 0.5 ml of inhibitor in buffer were then placed in the cuvette and the assay continued as above.

For the acetylcholinesterase experiments human erythrocytes were twice washed in 0.9% saline and then haemolysed by adding 100 μ l of packed cells to 10 ml of distilled water. After centrifugation this preparation was employed as the source of human acetylcholinesterase. To 1 ml of buffer containing varying concentrations of inhibitor, 200 μ l of haemolysate was added and the reaction started as indicated above by priming with 100 μ l of acetylthiocholine. For these experiments the acetylthiocholine-DTNB (reagent (ii), p. 106) was diluted 1 in 10 with phosphate buffer to give a final substrate concentration of 0.5 mM, optimal for acetylcholinesterase.

Although the level of non-enzymic hydrolysis was constant for each series of experiments a blank to monitor this was included in each rack of ten cuvettes.

Experimental Results

Preliminary experiments to establish optimal assay conditions by investigating the variation of enzyme activity with substrate concentration, buffer and pH, confirmed the lability of the thiocholine esters. The non-enzymic or spontaneous hydrolysis obviously increased in the order butyryl-, propionyl-, acetyl-, succinyl-, and while the percentage rate of hydrolysis remained sensibly constant

the absolute amount of product obviously increased with substrate concentration (Figure 65). The spontaneous hydrolysis also increased with pH (Figure 45). An observation from standard cysteine curves prepared at different pH values appeared to indicate that the absorption at 410 nm of the 5-thio-2-nitrobenzoate anion increased with pH. To check this 0.5 ml each of aqueous cysteine solution and DTNB solution were added to 3 ml of different buffers of varying pH values. Blanks with water substituted for cysteine solution were also prepared. Absorptions were recorded at different temperatures and at various time intervals. Typical results are illustrated in Figure 46.

These results confirmed the original observation and indicated that the increase in spontaneous hydrolysis with pH was in part due to the increased absorption of the indicator ion with pH. This also meant that the observed variation in enzyme activity with hydrogen ion concentration was similarly affected. This latter phenomenon and the non-enzymic hydrolysis of the thiocholine esters were largely the determinants in deciding to retain the buffer used in previous experiments, that is, 67 mM Sørensen phosphate buffer, pH 7.4 and substrate concentrations of 5 mM, 5 mM and 3 mM for acetyl-, propionyl- and butyrylthiocholine respectively at 37°C. These were the standard assay conditions employed in all later experiments. The correctness of this decision gained support from the work of Szasz (1968a) who, at 25°C, used 50 mM tris buffer and 2 mM butyrylthiocholine. This worker also recorded that the optimum for acetylthiocholine was "only reached at a concentration of

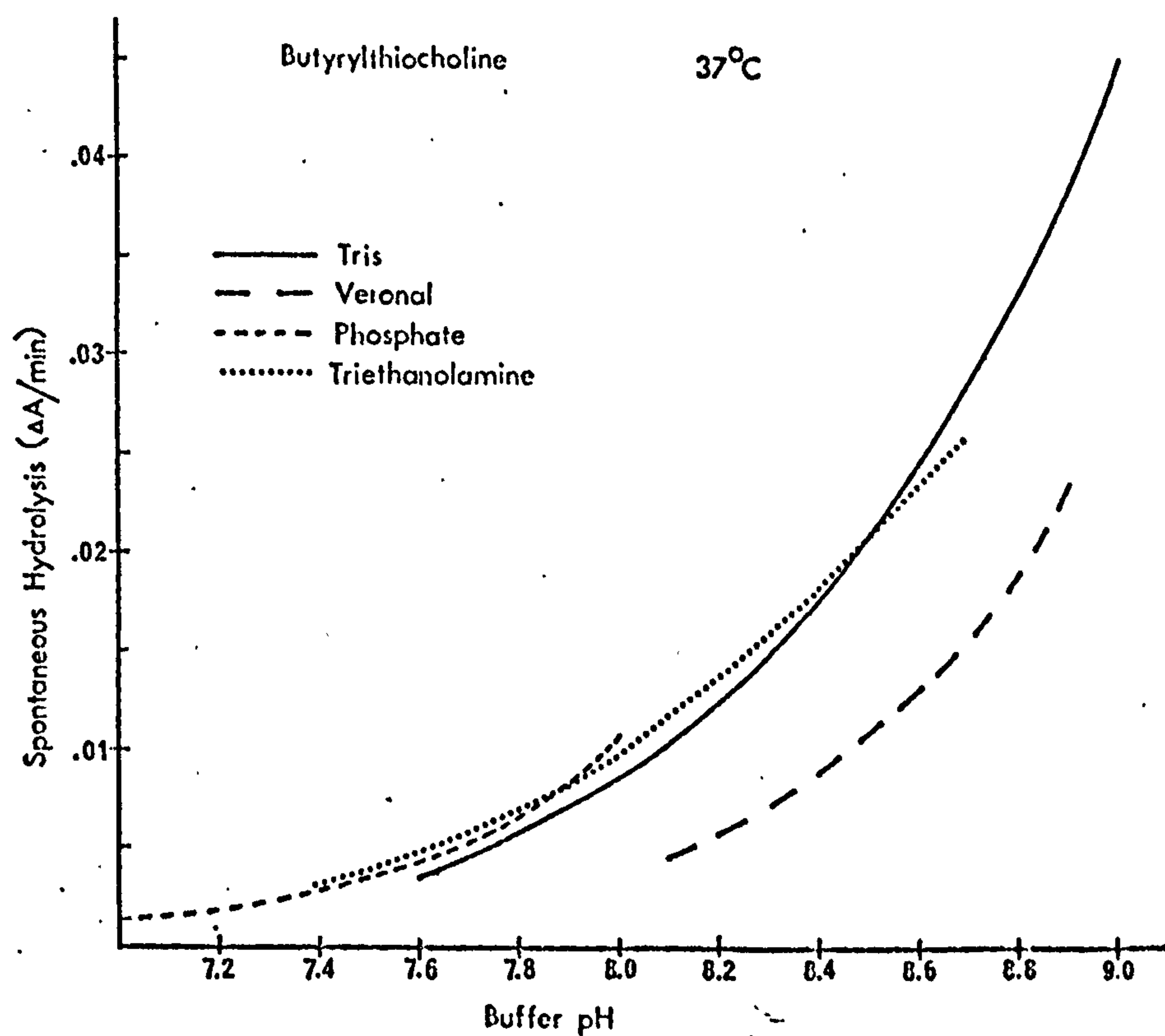


Figure 45. Variation of non-enzymic hydrolysis of butyrylthiocholine with pH and type of buffer.

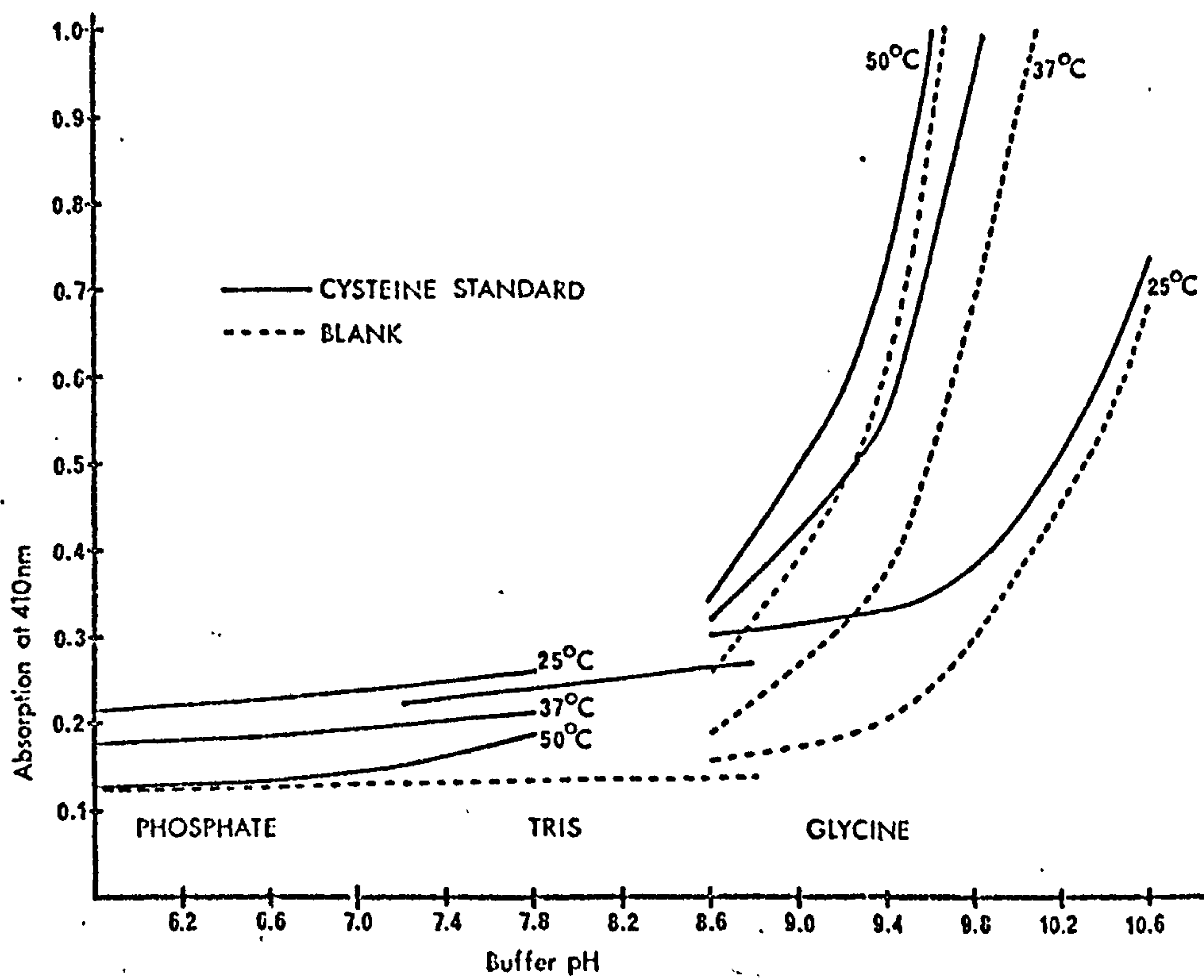


Figure 46. Variation in the absorption at 410 nm of the 5-thio-2-nitrobenzoate anion (Figure 42) with pH, type of buffer and temperature.

at least 5 mM. This increase in concentration does however raise considerably the autolysis of the substrate".

Consequently Szasz used a 2 mM concentration of acetylthiocholine at 25°C, thereby indicating similar experiences to those described above.

Experiments with succinyldithiocholine were disappointing due to the spontaneous hydrolysis at 37°C (Figure 65). In many cases particularly with sera from suxamethonium-sensitive phenotypes or with low normal activities, 90% or more of the recorded change in absorption was due to non-enzymic factors. Studies with succinyldithiocholine were not continued because of this lack of sensitivity in the assay.

Inhibition of the cholinesterase hydrolysis of the thiocholine esters was only examined with a view to establishing that the characteristics of the variants exhibited with benzoylcholine were also shown with these substrates. Figures 47, 48 and 49 illustrate the effects of increasing dibucaine concentration of the hydrolysis at 37°C of 5 mM acetylthiocholine. 5 mM propionylthiocholine and 3 mM butyrylthiocholine respectively by the six cholinesterase phenotypes studied. Similar curves were obtained using succinyldicholine, chloride and with certain anomalies, fluoride as shown in Figures 50 to 58.

To complete studies reported earlier (p. 83 et seq.) the inhibition of erythrocyte acetylcholinesterase hydrolysis of acetylthiocholine was also examined and the results with pancuronium and tubocurarine are shown in Figures 59 and 60 respectively. Gallamine inhibition was also attempted in

these acetylcholinesterase experiments but addition of the substrate-DTNB solution produced such an intense yellow colour that recording of reaction rates was not possible. Although the May and Baker leaflet on Flaxedil gave no indication as to the possible cause of this phenomenon the British Pharmacopoeia (p.334) indicates, that for injection, gallamine triethiodide is dissolved in 0.2% w/v sodium sulphite.

It was evident at an early stage of preliminary studies that the relative rates of hydrolysis of the different substrates varied with the serum phenotype. This had also been observed with a wide range of choline esters (Davies, Marton and Kalow, 1960) with acetylcholine and benzoylcholine (Rubinstein and Dietz, 1963) and with acetylcholine, benzoylcholine, procaine and tetracaine (Smith and Foldes, 1972). At most these studies only compared the usual and atypical homozygotes and the heterozygote. To extend these observations sera from 34 usual homozygotes, 5 usual/fluoride-resistant heterozygotes, 21 usual/atypical heterozygotes, 2 fluoride-resistant homozygotes, 4 atypical/fluoride-resistant heterozygotes and 11 atypical homozygotes were assayed under the prescribed conditions at 37°C. The best differentiation was obtained with acetylthiocholine and butyrylthiocholine and is shown in Figure 61.

The results of the temperature studies with the three thiocholine ester substrates and six phenotypes are illustrated in Figures 62, 63 and 64. In these figures the spontaneous hydrolysis (Figure 65) is allowed for but no account is taken of the increase in absorption with tempera-

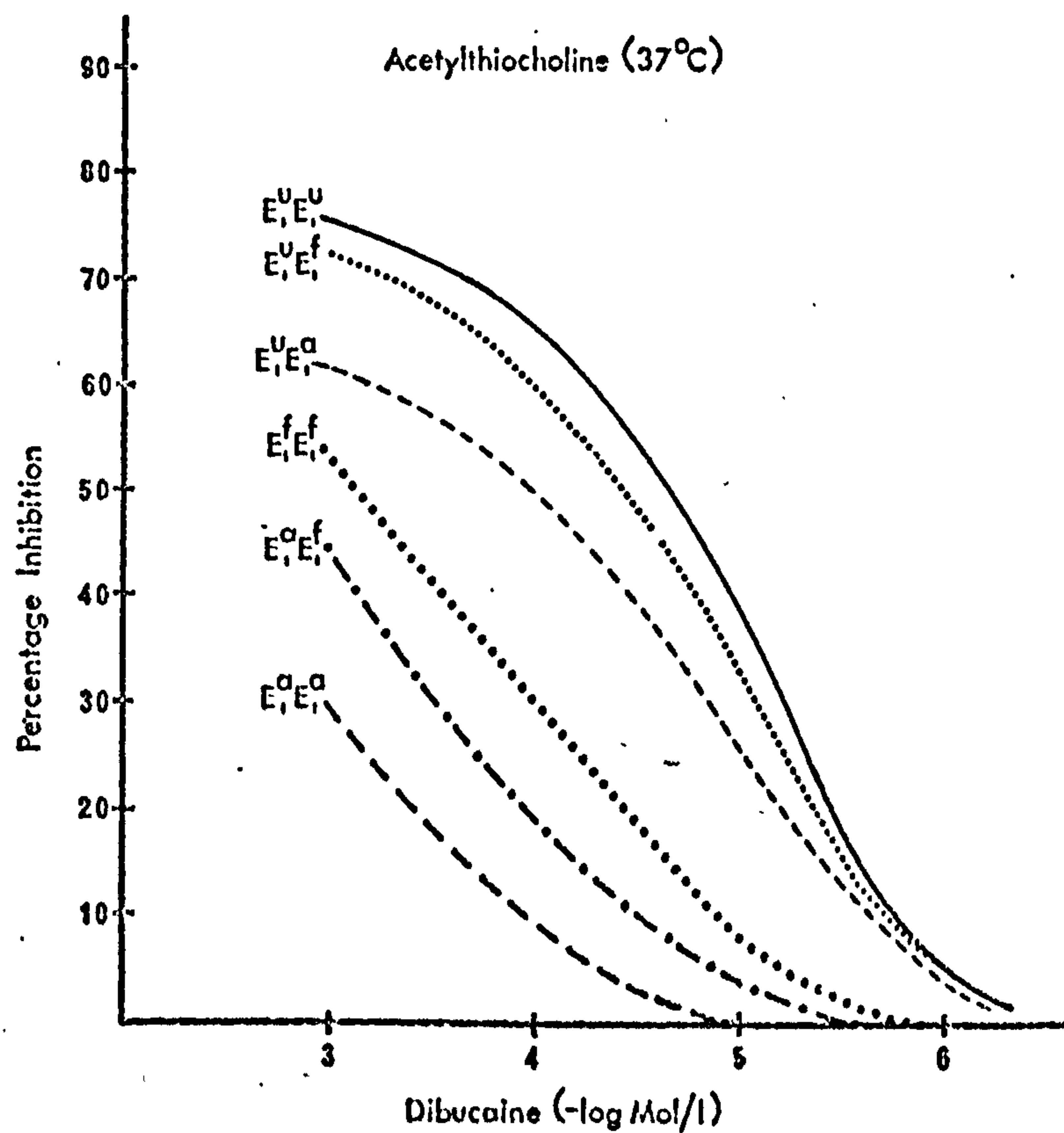


Figure 47. Dibucaine inhibition of acetylthiocholine hydrolysis by cholinesterase phenotypes at 37°C, with a reaction concentration of 5 mM acetylthiocholine in 67 mM Sørensen phosphate buffer pH 7.4.

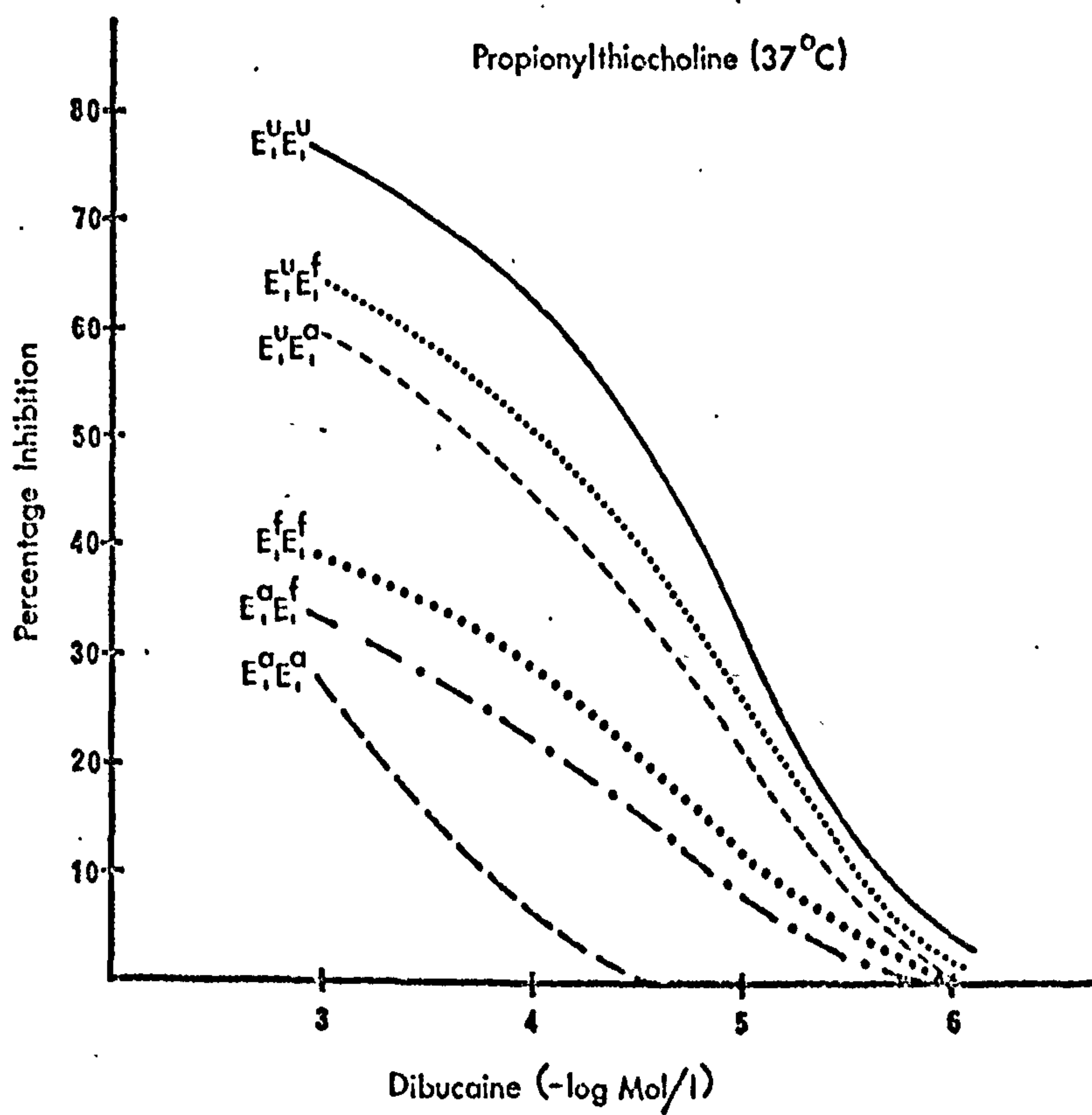


Figure 48. Dibucaine inhibition of propionylthiocholine hydrolysis by cholinesterase phenotypes at 37°C with a reaction concentration of 5 mM propionylthiocholine in 67 mM Sørensen phosphate buffer, pH 7.4.

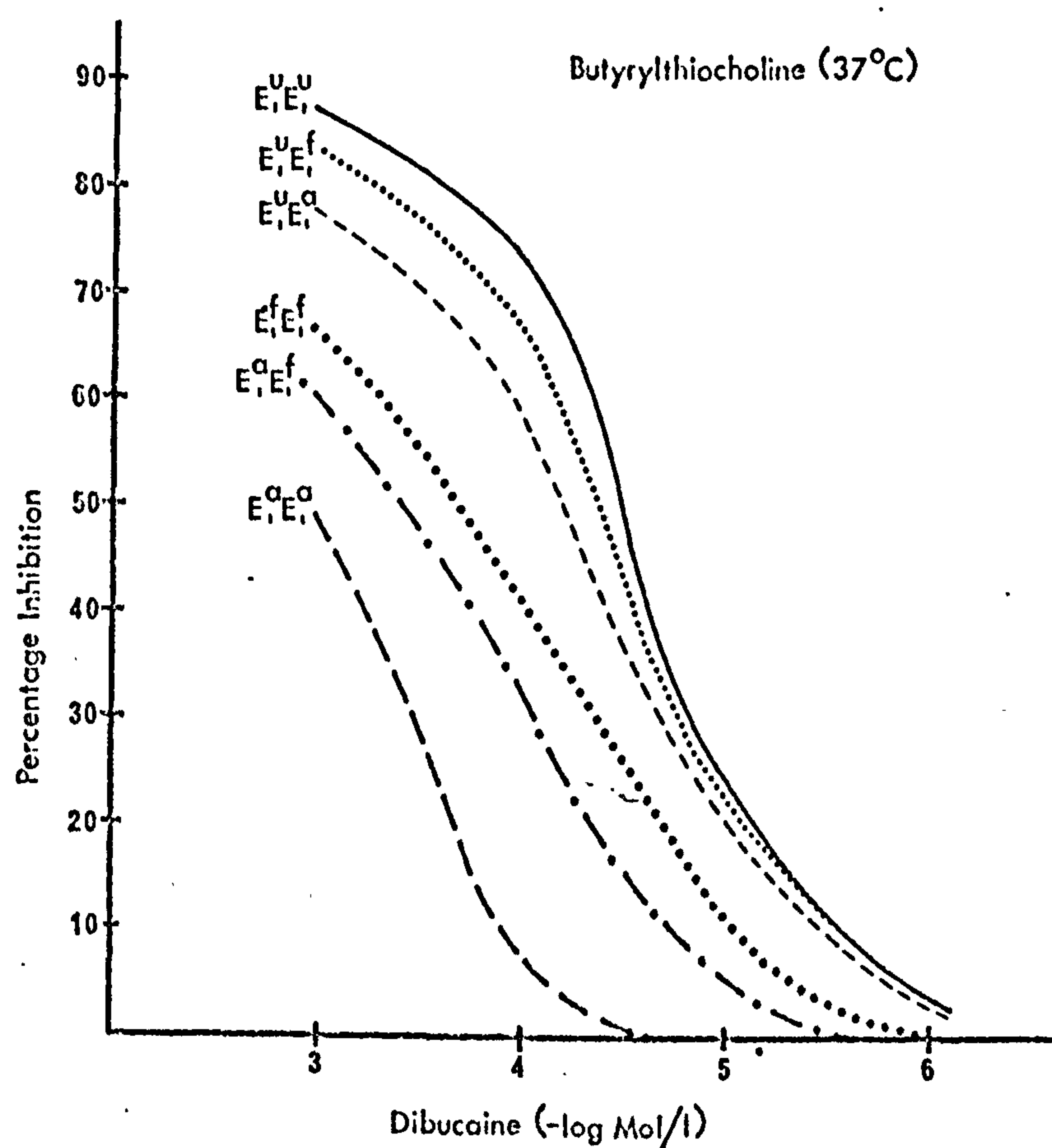


Figure 49. Dibucaine inhibition of butyrylthiocholine hydrolysis by cholinesterase phenotypes at 37°C with a reaction concentration of 3 mM butyrylthiocholine in 67 mM Sørensen phosphate buffer, pH 7.4.

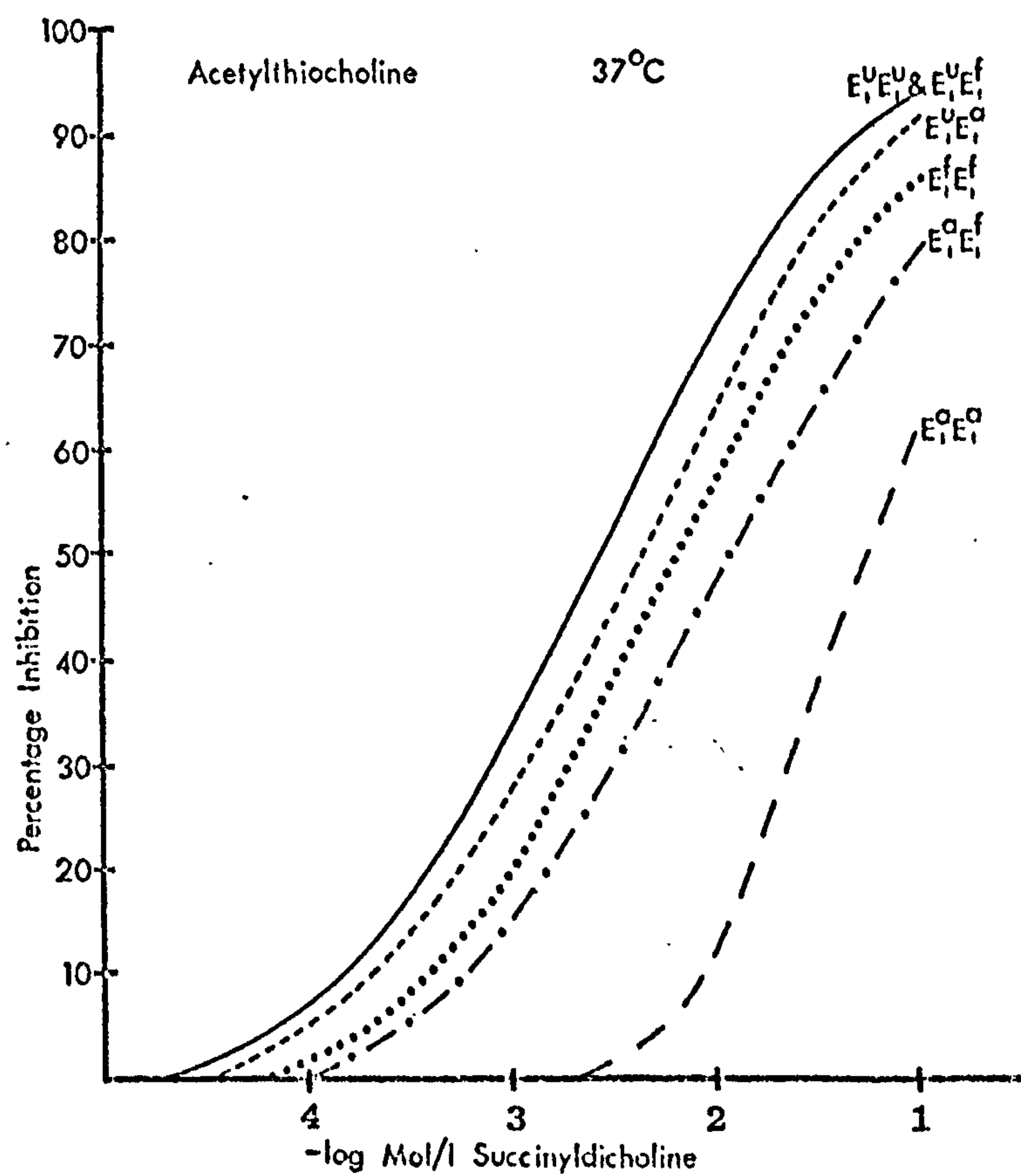


Figure 50. Succinylcholine inhibition of acetylthiocholine hydrolysis by cholinesterase phenotypes at 37°C with a reaction concentration of 5 mM acetylthiocholine in 67 mM Sørensen phosphate buffer, pH 7.4.

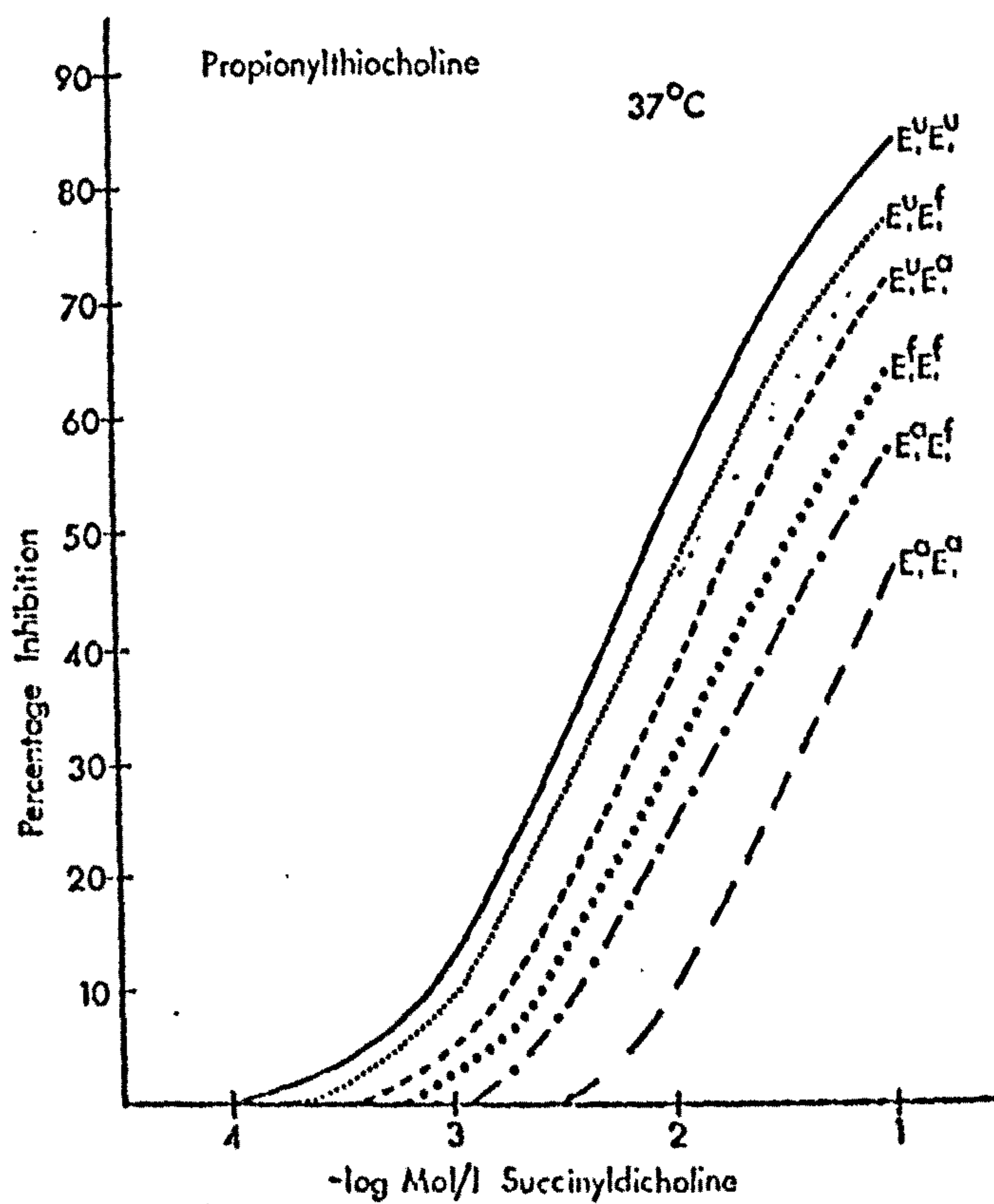


Figure 51. Succinylcholine inhibition of propionylthiocholine hydrolysis by cholinesterase phenotypes at 37°C with a reaction concentration of 5 mM propionylthiocholine in 67 mM Sørensen phosphate buffer, pH 7.4.

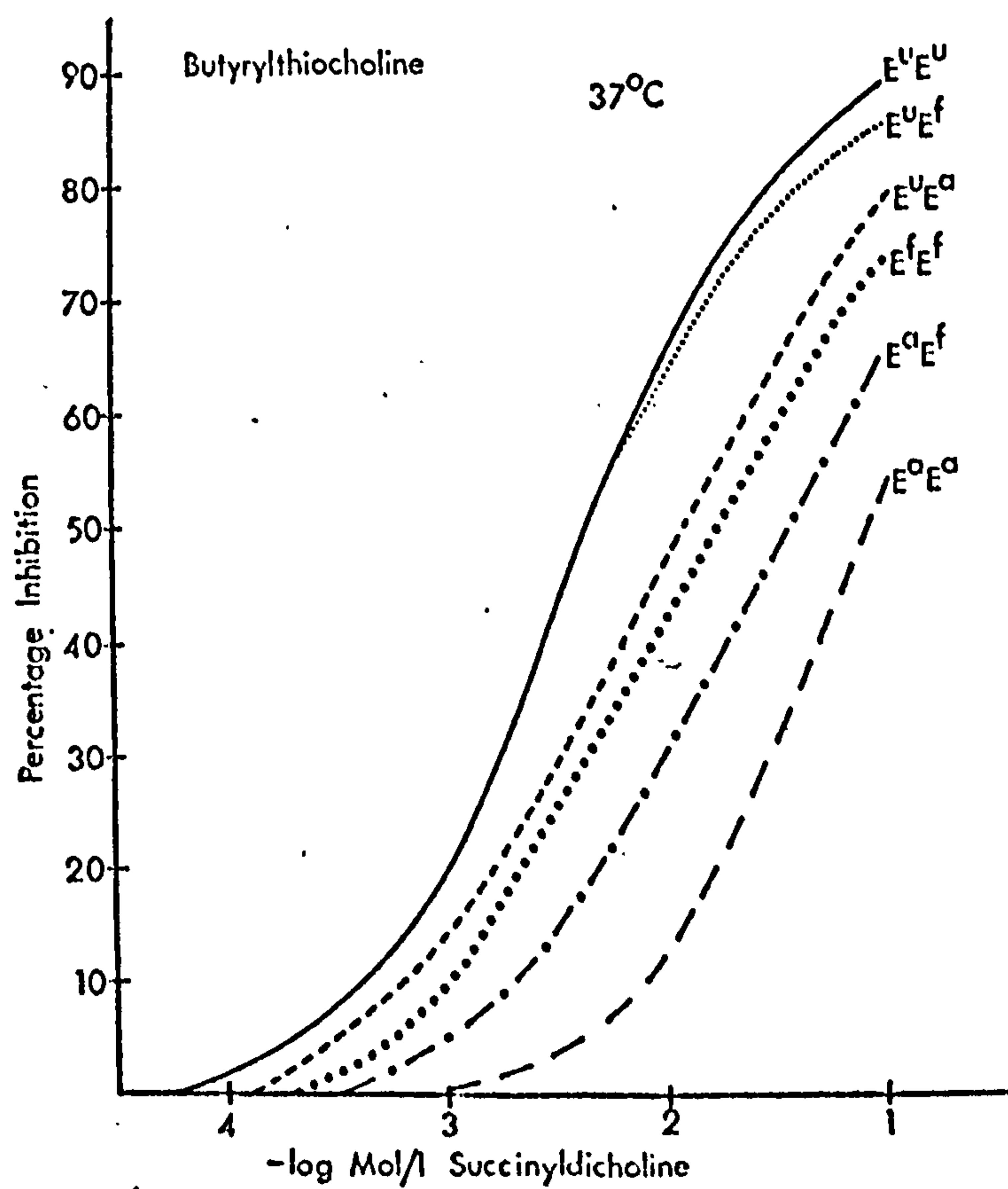


Figure 52. Succinylcholine inhibition of butyrylthiocholine hydrolysis by cholinesterase phenotypes at 37°C with a reaction concentration of 3 mM butyrylthiocholine in 67 mM Sørensen phosphate buffer, pH 7.4.

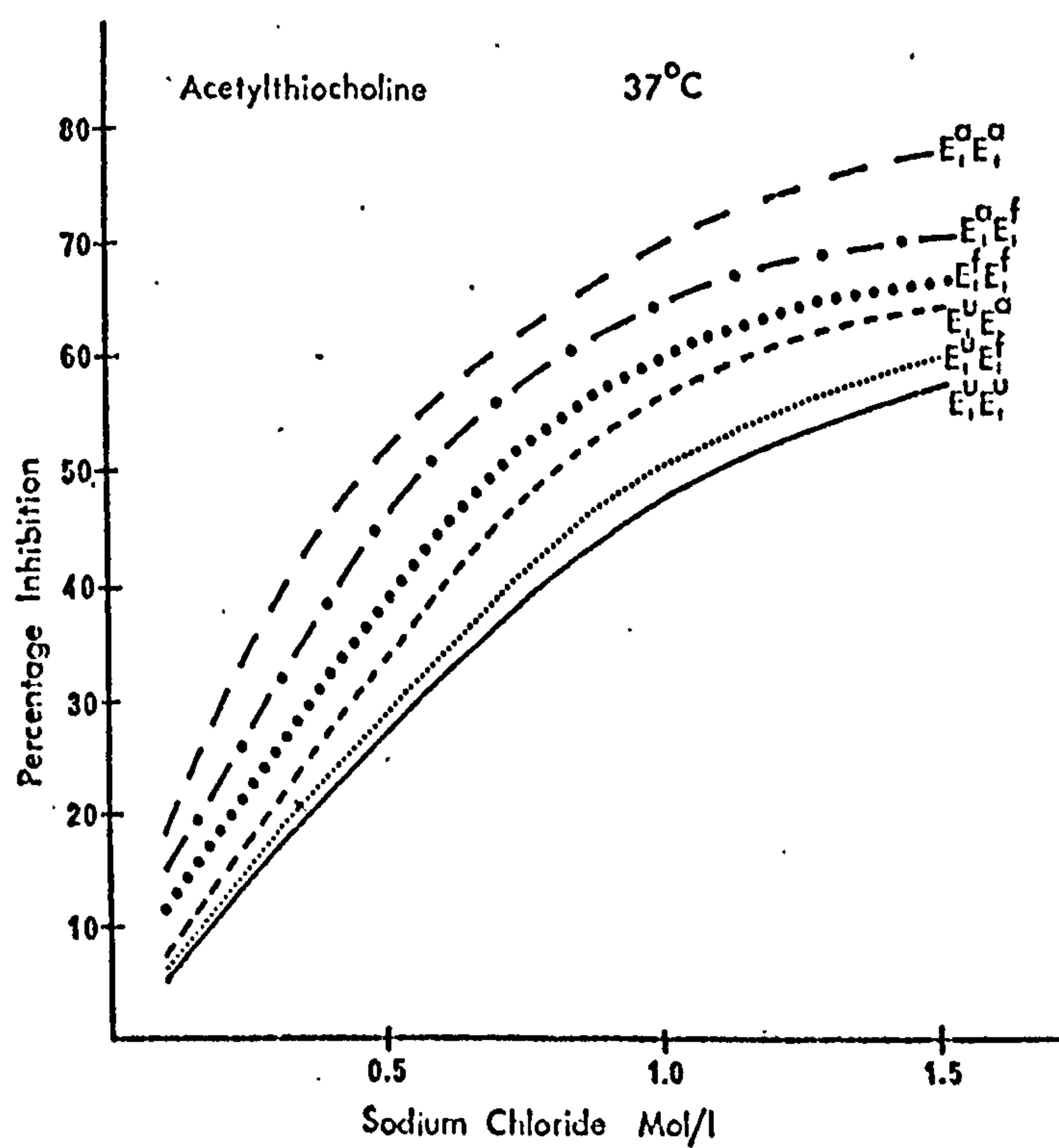


Figure 53. Sodium chloride inhibition of acetylthiocholine hydrolysis by cholinesterase phenotypes at 37°C with a reaction concentration of 5 mM acetylthiocholine in 67 mM Sørensen phosphate buffer, pH 7.4.

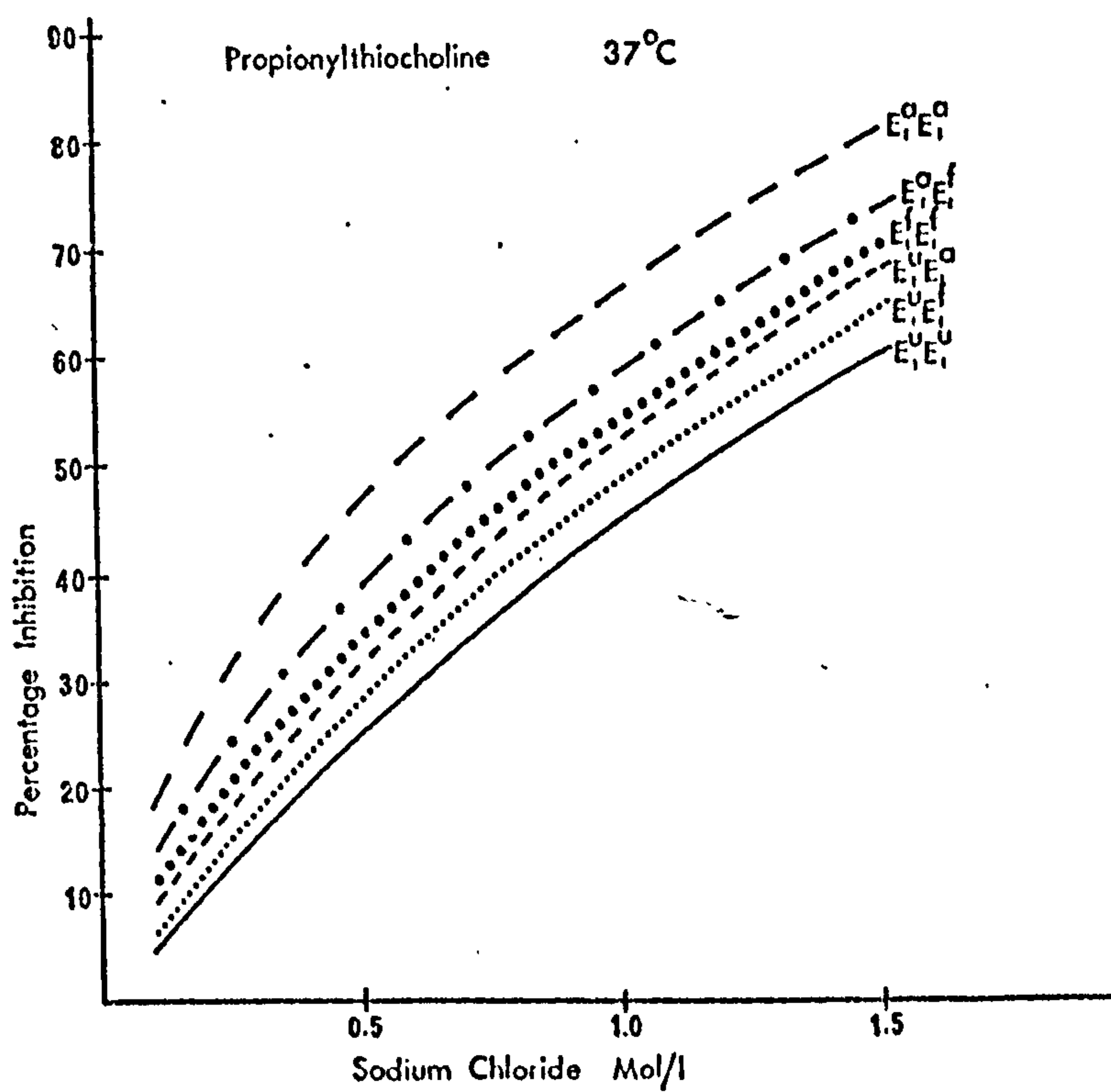


Figure 54. Sodium chloride inhibition of propionylthiocholine hydrolysis by cholinesterase phenotypes at 37°C with a reaction concentration of 5 mM propionylthiocholine in 67 mM Sørensen phosphate buffer, pH 7.4.

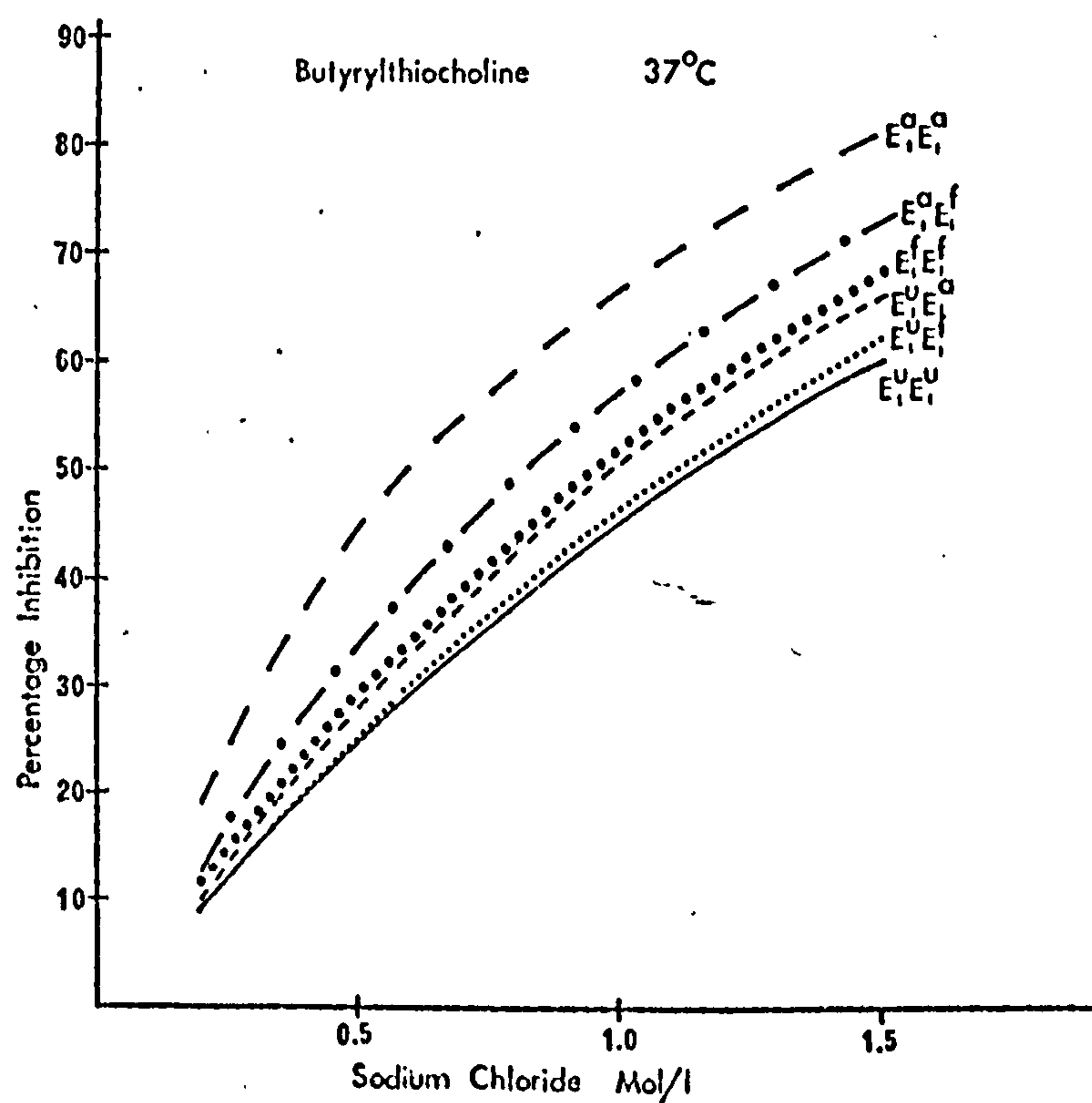


Figure 55. Sodium chloride inhibition of butyrylthiocholine hydrolysis by cholinesterase phenotypes at 37°C with a reaction concentration of 3 mM butyrylthiocholine in 67 mM Sørensen phosphate buffer, pH 7.4.

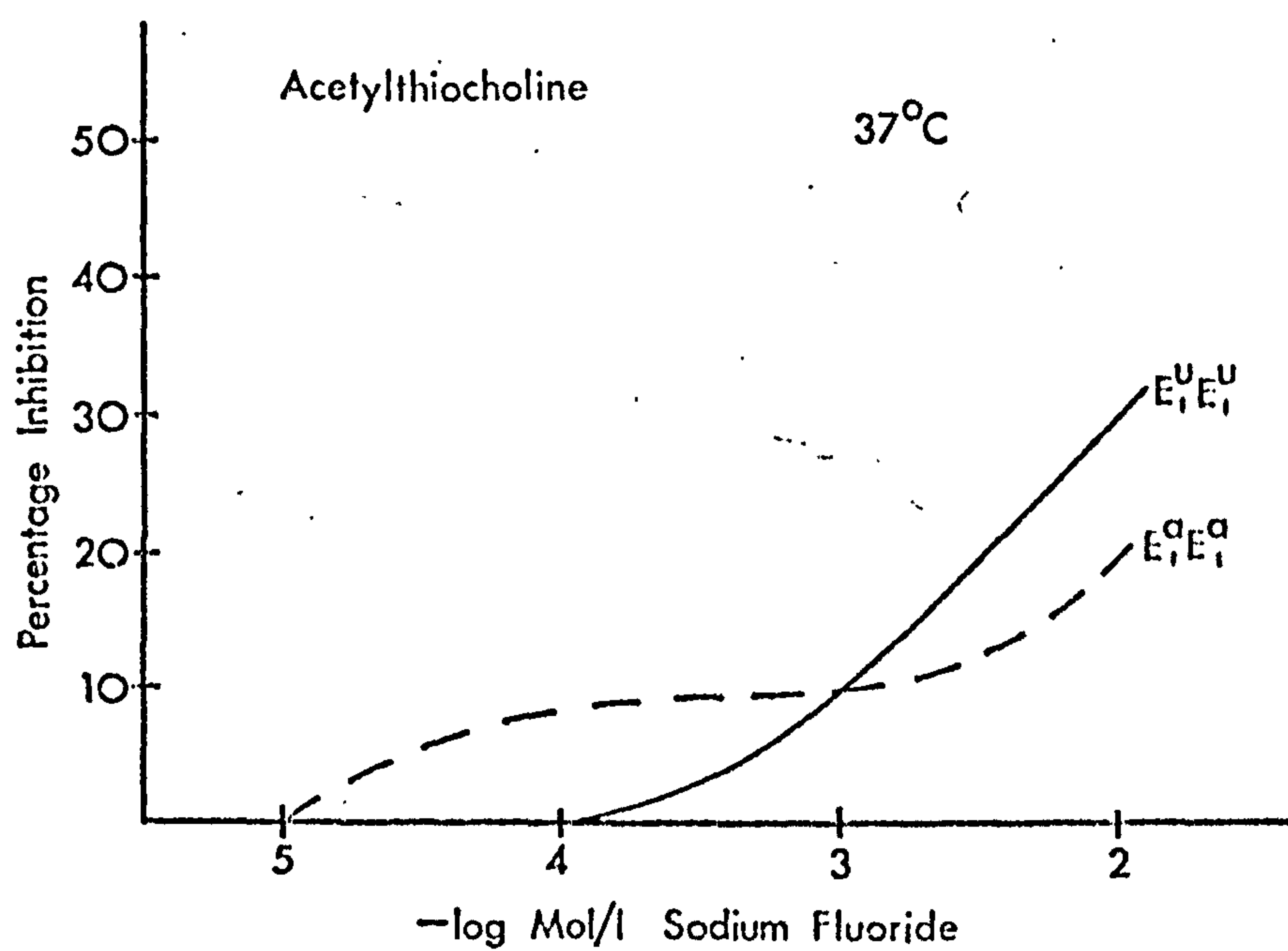


Figure 56. Fluoride inhibition of acetylthiocholine hydrolysis by cholinesterase phenotypes at 37°C with a reaction concentration of 5 mM acetylthiocholine in 67 mM Sørensen phosphate buffer, pH 7.4. The curves for the other phenotypes lie between those extremes shown and are omitted for clarity of presentation.

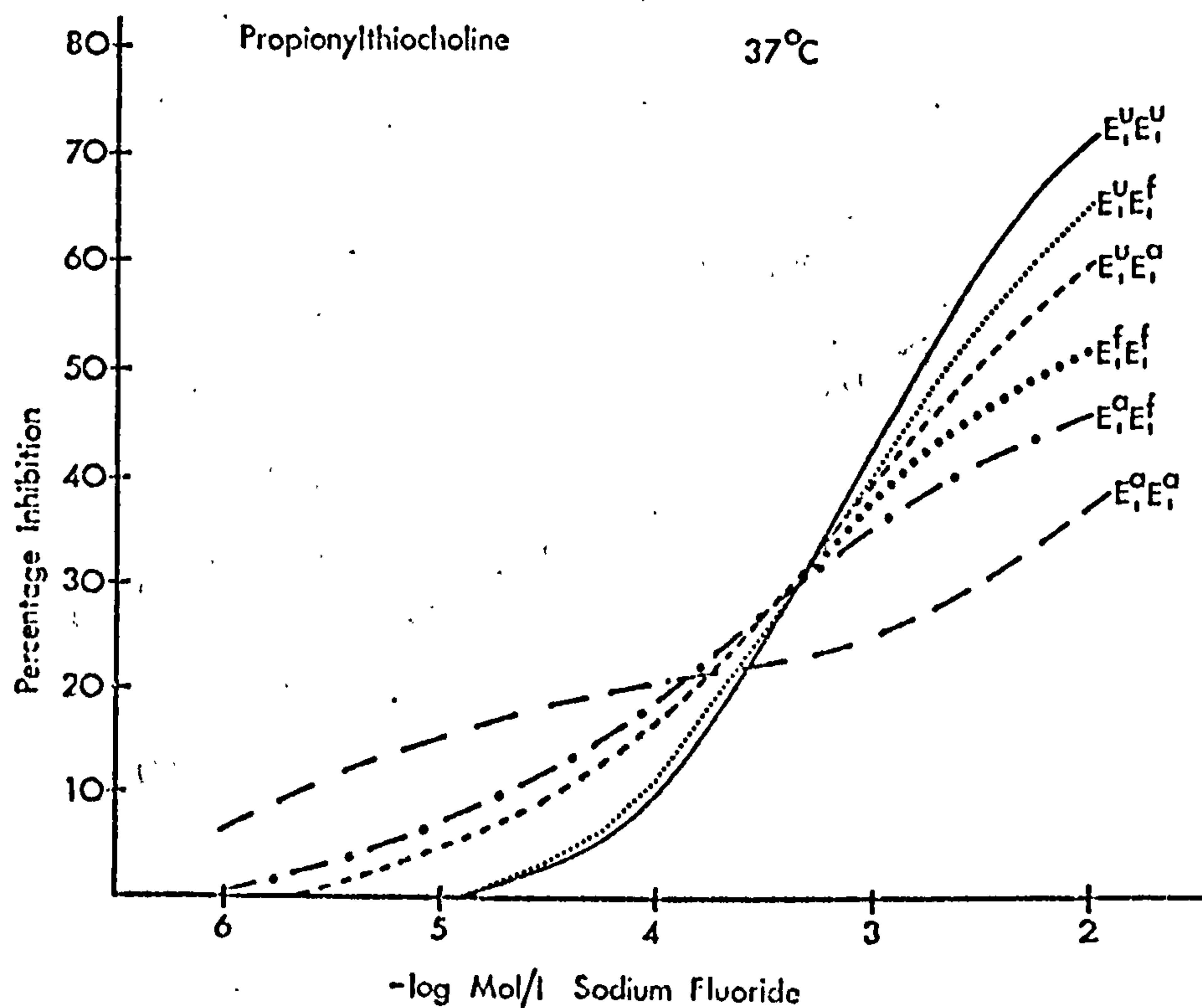


Figure 57. Fluoride inhibition of propionylthiocholine hydrolysis by cholinesterase phenotypes at 37°C with a reaction concentration of 5 mM propionylthiocholine in 67 mM Sørensen phosphate buffer, pH 7.4.

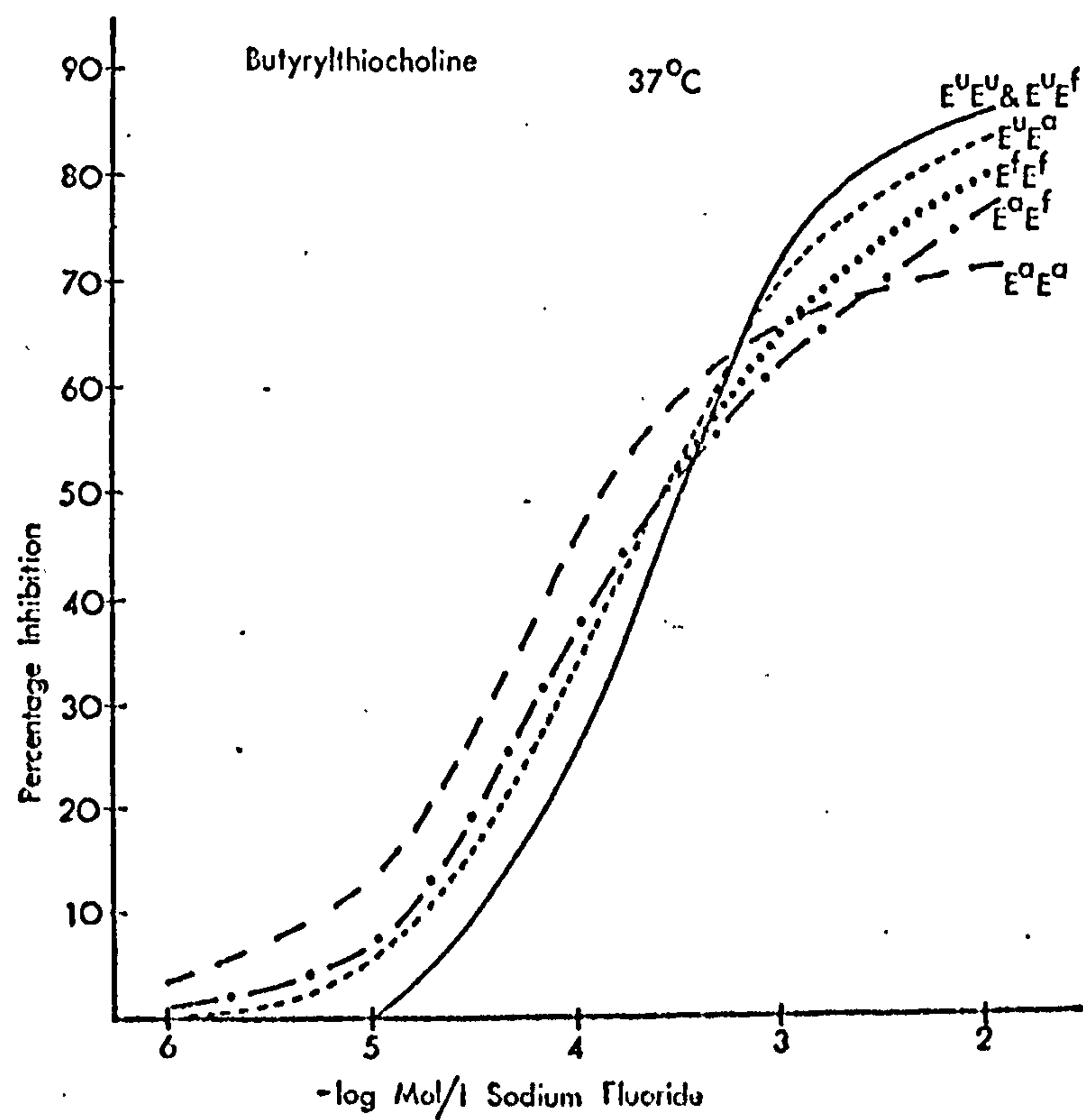


Figure 58. Fluoride inhibition of butyrylthiocholine hydrolysis by cholinesterase phenotypes at 37°C with a reaction concentration of 3 mM butyrylthiocholine in 67 mM Sörensen phosphate buffer, pH 7.4.

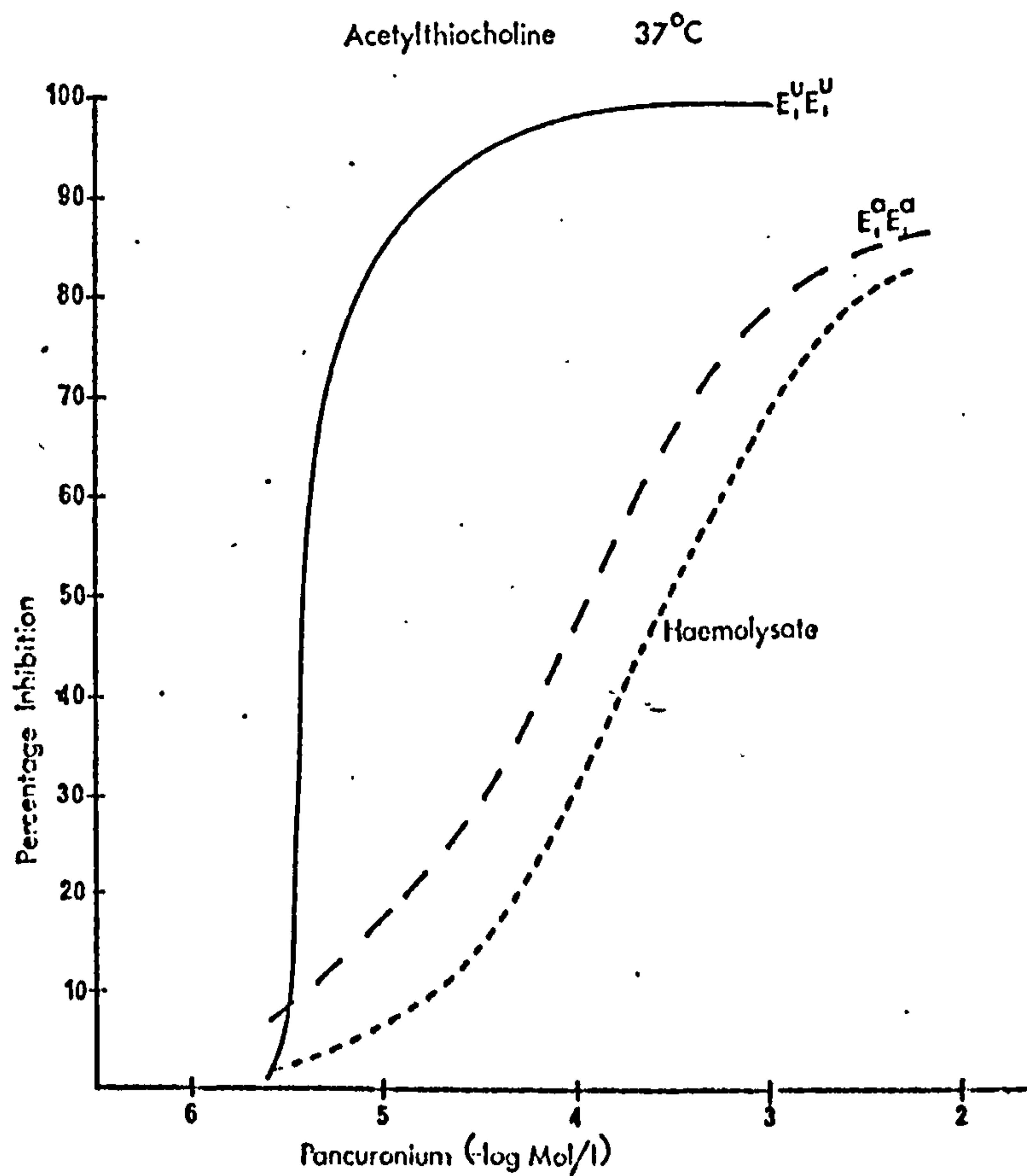


Figure 59. Pancuronium inhibition of acetylthiocholine hydrolysis by serum cholinesterases and erythrocyte acetylcholinesterase at 37°C. For the cholinesterase assays a reaction concentration of 5 mM acetylthiocholine and for the acetylcholinesterase studies of 500 μ M acetylthiocholine in 67 mM Sørensen phosphate buffer, pH 7.4 were employed.

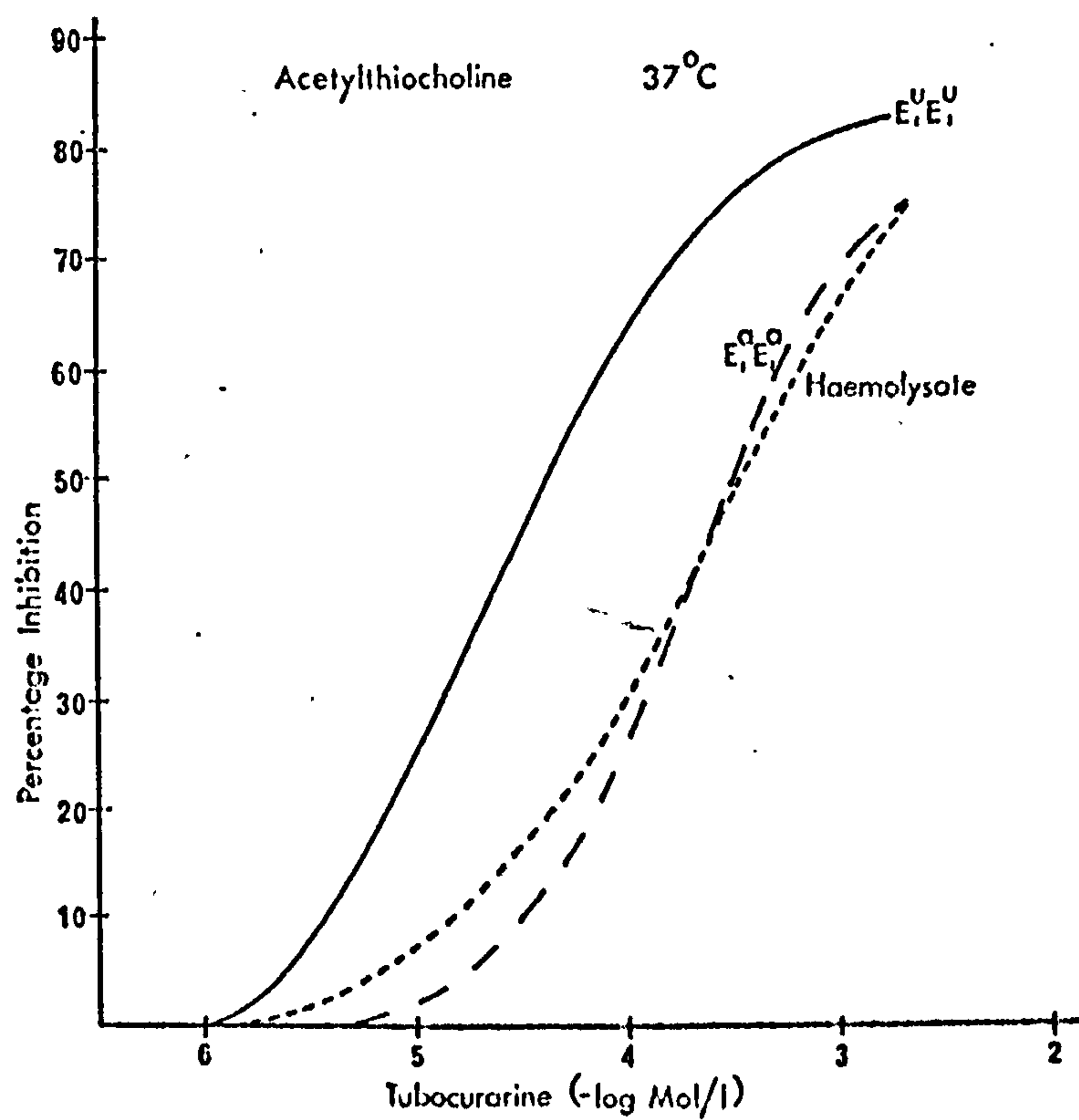


Figure 60. Tubocurarine inhibition of acetylthiocholine hydrolysis by serum cholinesterases and erythrocyte acetylthiocholinesterase at 37°C. For the cholinesterase assays a reaction concentration of 5 mM acetylthiocholine and for the acetylcholinesterase estimations of 500 μM acetylthiocholine in 67 mM Sørensen phosphate buffer, pH 7.4 were employed.

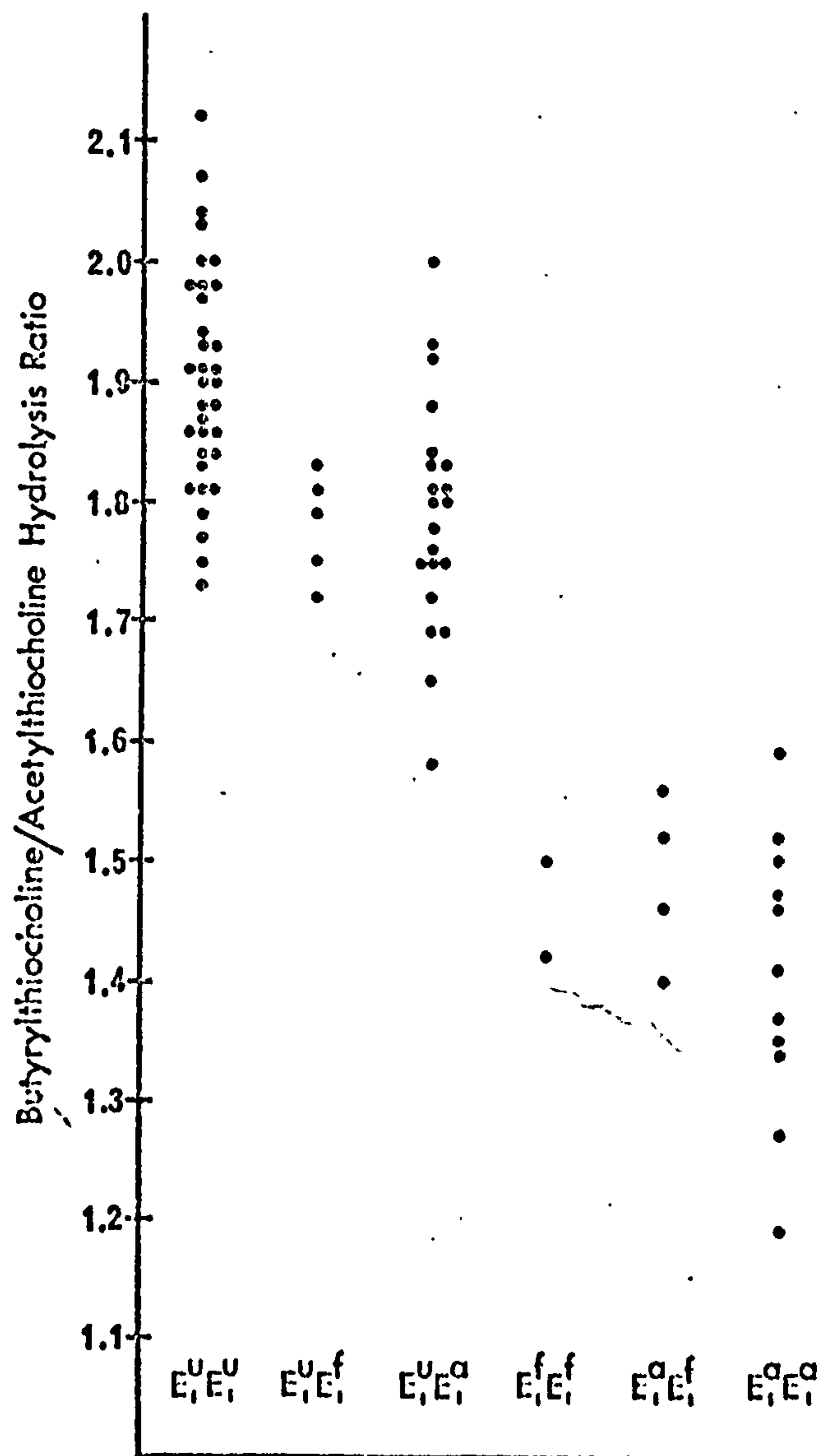


Figure 61. Ratios of activities at 37°C with 3 mM butyrylthiocholine and 5 mM acetylthiocholine for serum cholinesterase phenotypes.

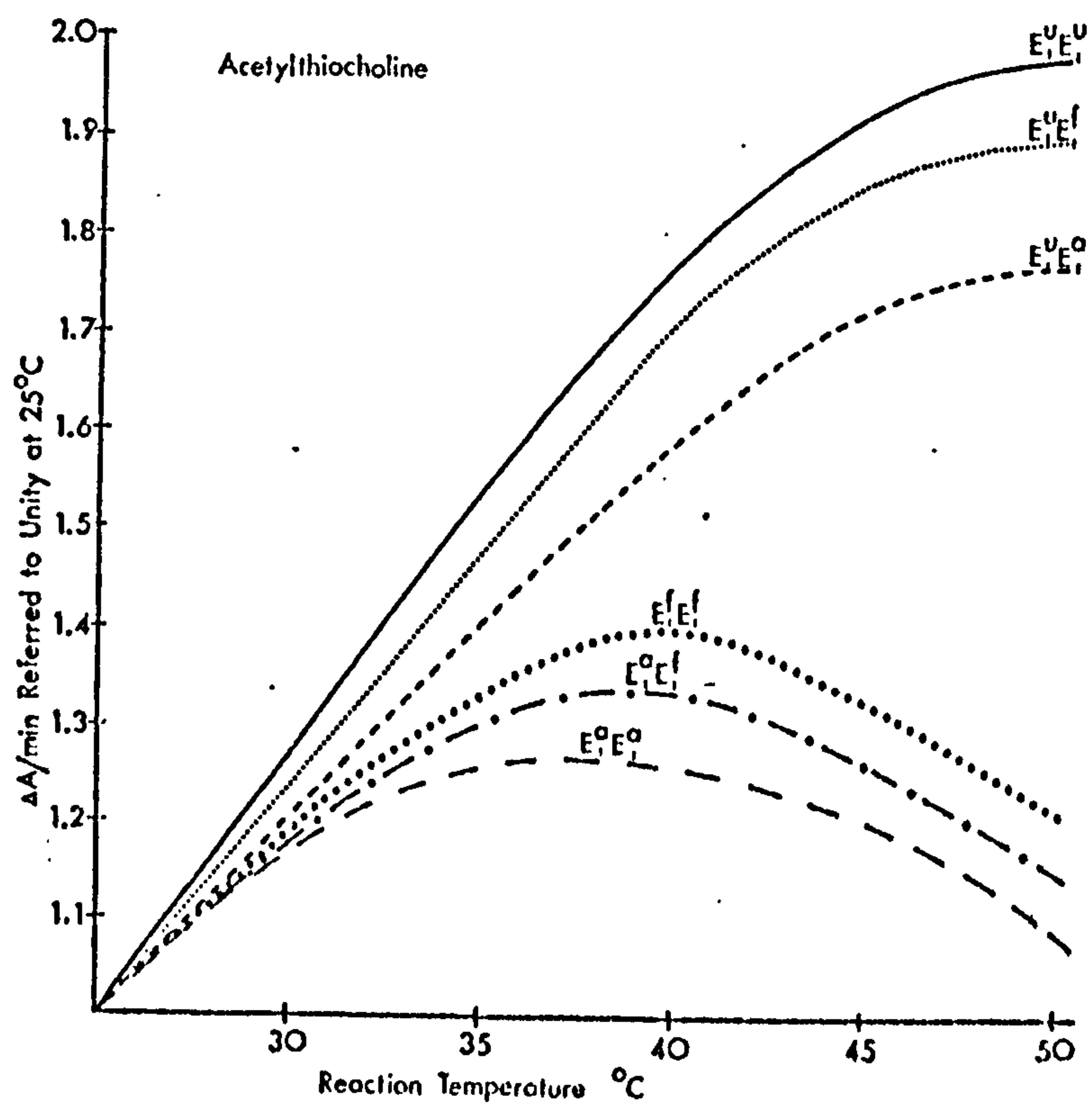


Figure 62. Temperature/activity curves of serum cholinesterase phenotypes with a 5 mM acetylthiocholine substrate and referred to unity at 25°C.

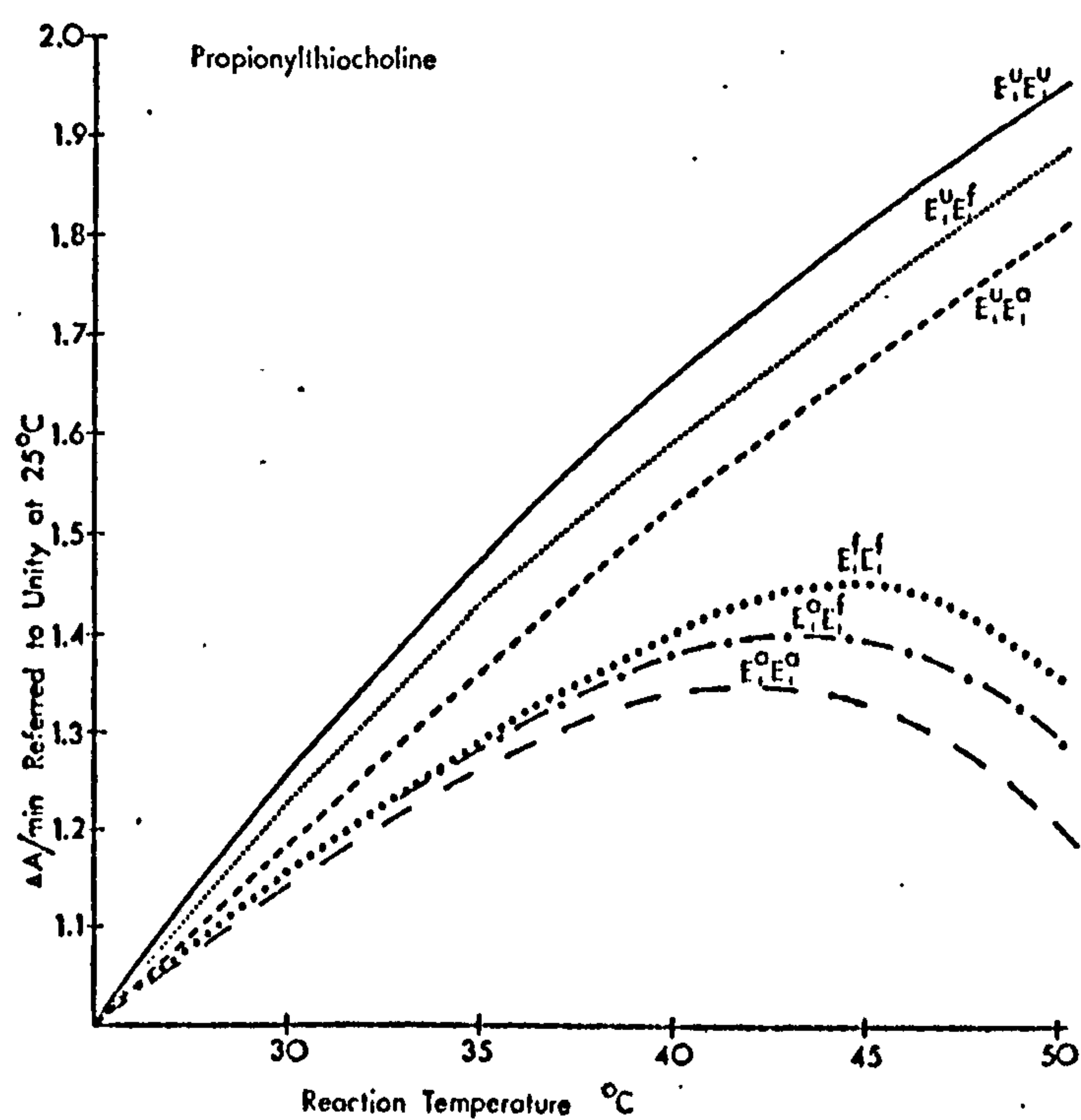


Figure 63. Temperature/activity curves of serum cholinesterase phenotypes with a 5 mM propionylthiocholine substrate and referred to unity at 25°C.

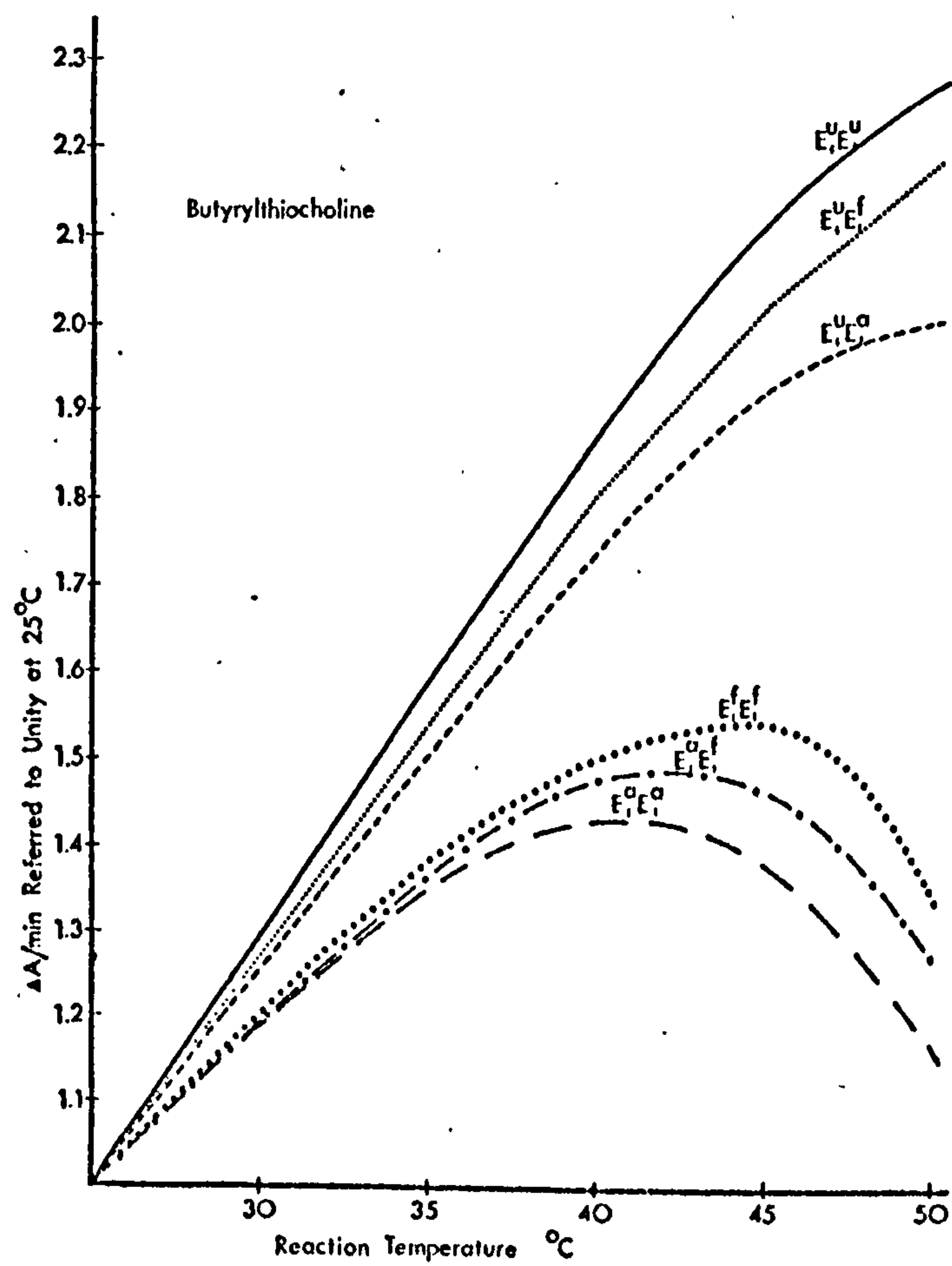


Figure 64. Temperature/activity curves of serum cholinesterase phenotypes with a 3 mM butyrylthiocholine substrate and referred to unity at 25°C.

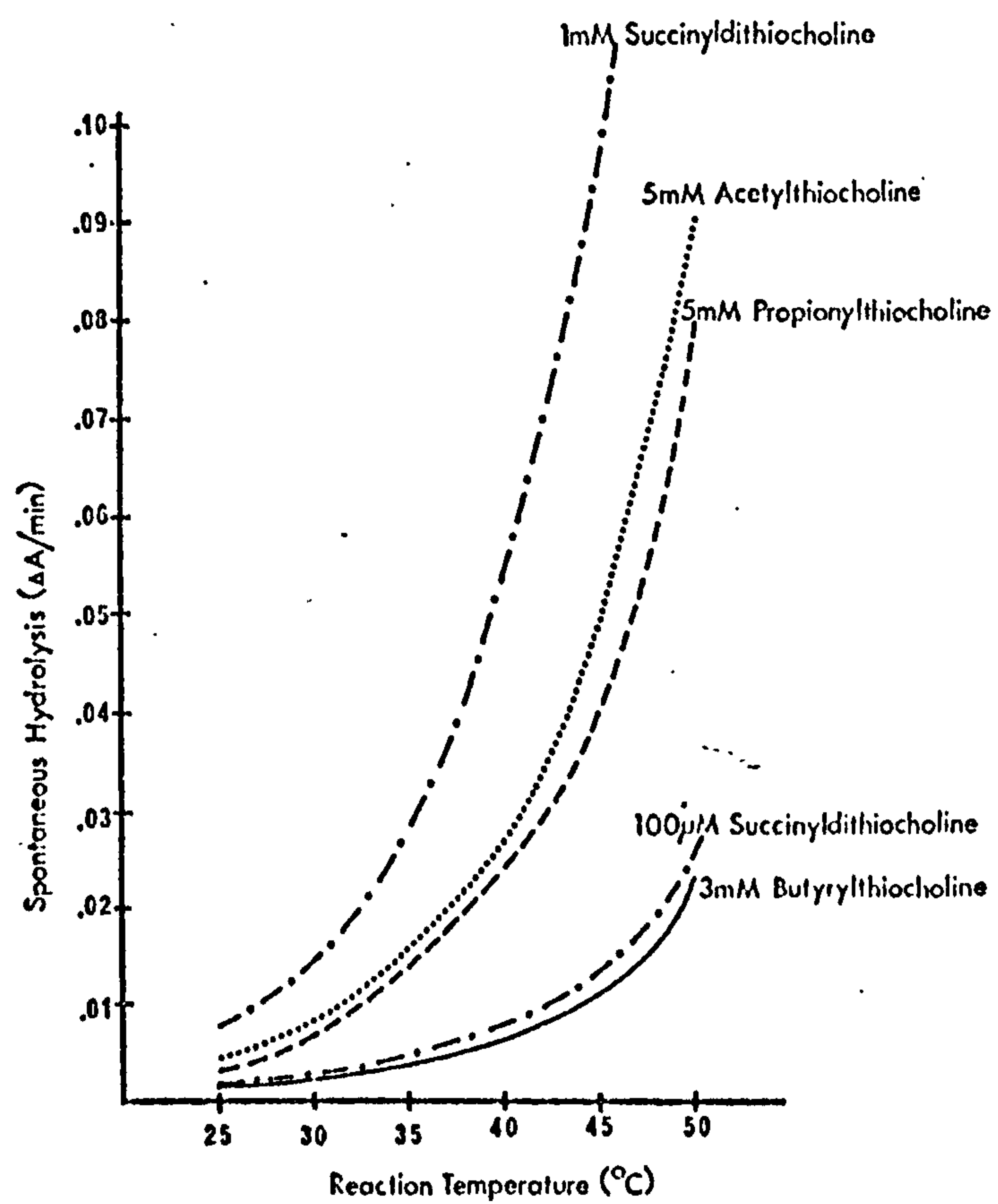


Figure 65. Variation of the non-enzymic hydrolysis of thiocholine esters with reaction temperature.

ture of the indicator ion (Figure 46).

Discussion

The non-enzymic hydrolysis of the thiocholine esters varies with the hydrogen ion concentration (Figure 45), substrate concentration and reaction temperature (Figure 65) and even the absorption of the indicator ion, 5-thio-2-nitrobenzoate, varies with pH and temperature (Figure 46). These facts, some apparently unappreciated by other workers, make this assay less ideal and reliable than that based on benzoylcholine hydrolysis.

Interpretation of results from benzoylcholine hydrolysis however must always be viewed from the background knowledge that this ester is hardly acceptable as a physiological substrate. Nonetheless if similar behaviour is exhibited by other substrates with close chemical affinity to naturally occurring esters then it may be concluded that these interpretations are real and valid. Therefore despite the doubts engendered by the preliminary investigations into the assay principle, the study was continued albeit bearing in mind that all that was sought or expected was generally confirmatory evidence. As a consequence no absolute values were recorded in these studies, only relative figures such as percentages and ratios.

The inhibition studies with dibucaine, succinylcholine and chloride amply provided the confirmation that these substances reacted with the enzyme protein. For dibucaine the concentration giving optimal differentiation between the phenotypes was 10^{-4} M with all three substrates. For acetylthiocholine and propionylthiocholine this gave

inhibitions about 65%, 30% and >10% for the homozygous phenotypes $E_1^u E_1^u$, $E_1^f E_1^f$ and $E_1^a E_1^a$ respectively (Figures 47 and 48. With butyrylthiocholine (Figure 49) these values become 75%, 40% and 10%. The difference in these inhibitions does not necessarily betoken a contrast in the handling of the substrates but is probably related to their concentrations, 5 mM for the acetyl and propionyl esters, 3 mM for butyrylthiocholine. When the dibucaine concentration is reduced proportionally to 6×10^{-5} M the inhibitions obtained with butyrylthiocholine are identical to those obtained at 10^{-4} M with the other two substrates. This is the expected behaviour of a simple competitive inhibitor.

The improved differentiation of the fluoride-resistant homozygote from other phenotypes is probably not a reflection of its behaviour in the presence of thiocholine ester substrates but rather of the reaction temperature, 37°C. It was noted earlier (Figure 35) that the inhibitions of the fluoride-resistant homozygote were more sensitive to temperature change than the other phenotypes and at 37°C yielded inhibitions more consistent with its known suxamethonium sensitivity (p. 99).

Succinylcholine is also a competitive inhibitor and its behaviour is therefore unexpected. The inhibitor concentration giving maximum differentiation is 10 mM at which the atypical homozygote is inhibited 10% irrespective of substrate. Under the same conditions the usual homozygote is inhibited 70% with acetylthiocholine, 55% with propionylthiocholine and 65% with butyrylthiocholine. Proportional adjustment of succinylcholine concentration to 6 mM reduces

the butyrylthiocholine inhibition to 55%. It is interesting to note that the optimal differentiating concentrations for both dibucaine and succinylcholine are tenfold greater than those employed with benzoylcholine. Although not directly comparable because of the different substrate concentrations employed and the use of tris buffer of pH 7.2 similar results to those described here for dibucaine and succinylcholine were reported by Garry (1971).

With sodium chloride the differential inhibition of phenotypes is much reduced and is fairly constant between 0.4M and 1.5M. Again the loci for the propionyl and butyryl esters are very similar with acetylthiocholine giving a slightly different picture and better segregation. Nonetheless at 0.4M sodium chloride the usual homozygote is inhibited about 20% and the atypical homozygote about 45% with all three substrates.

With sodium fluoride the most bizarre results are obtained with distinct progressive variations depending upon the substrate. The degree of inhibition for all phenotypes increases with substrate chain length. At lower inhibitor concentrations the relative phenotype inhibitions are the reverse of that found with a benzoylcholine substrate but with increasing concentration of fluoride this becomes inverted. The first part of these curves with the atypical variant more inhibited than the usual enzyme is illustrated by Garry (1971) using a butyrylthiocholine substrate. This worker did not continue the fluoride concentration beyond 5×10^{-4} M at which he was obtaining almost complete inhibition of all phenotypes. The effect of other inhibitors

were examined by Garry at 37°C but strangely the fluoride experiments were conducted at 25°C. Heilbronn (1965) had already noted that fluoride inhibition of butyrylcholine hydrolysis increased with lowering of temperature as noted in this study with a benzoylcholine substrate (Figure 39).

Garry (1971) did employ higher concentrations of fluoride up to 10^{-2} M but in these experiments in tris buffer. At these higher concentrations the usual enzyme was inhibited to a greater extent than the atypical variant, the inversion to the situation found with benzoylcholine substrate. This inversion was attributed to the effect of the change in buffer from phosphate to tris. Certainly in tris buffer the inhibitory effect of fluoride is much reduced as well as the relative phenotype inhibitions inverted to that found in phosphate buffer. This phenomenon is made use of by Garry, Owen and Lubin (1972) to differentiate the fluoride-resistant variants by duplicate assays at 25°C in phosphate and tris buffer, with and without addition of fluoride. Obviously from the results obtained in this study (Figure 58) the same differentiation could be obtained at 37°C in phosphate buffer alone at two fluoride concentrations, say 10^{-4} M and 10^{-2} M with a butyrylthiocholine substrate.

In the Selected Method of the American Association of Clinical Chemists (Dietz, Rubinstein and Lubrano, 1973) a 2 mM propionylthiocholine substrate, with 4 mM sodium fluoride, phosphate buffer, pH 7.6 and reaction temperature of 37°C is recommended. These conditions yield highest percentage inhibitions with overlapping ranges for the usual (78-81%) and atypical homozygotes (82-86%) and the heterozygote

(78-82%), lowest values of 54 and 65% for the fluoride-resistant homozygote and 67-69% for the atypical/fluoride-resistant heterozygote. Obviously the conditions chosen are close to the point of inhibition inversion and incredibly in an official Selected Method this would appear not to have been appreciated.

In unrecorded experiments on benzoylcholine hydrolysis it was found that at the inhibitor concentrations routinely employed (page 43) increasing the substrate concentration from 50 μ M to 100 μ M reduced the inhibition by the competitive inhibitors, dibucaine and succinylcholine by about 10% but increased the fluoride number by about 10. The complexity of fluoride inhibition had been appreciated therefore before these thiocholine ester studies. The increase in inhibition with increase in substrate concentration had given rise to the idea that fluoride acted by forming a complex with the substrate. That this complex might be thermolabile would be in accord with the pronounced decrease in inhibition with increase in reaction temperature (Figure 39). This simple hypothesis might also be in keeping with the difference in degree of inhibition experienced with the different thiocholine substrates (Figures 56, 57, 58). This last phenomenon had already been reported by Heilbronn (1965) who noted that sensitivity to fluoride inhibition decreased in the order, benzoylcholine, butyrylcholine, acetylcholine. Cimasoni (1966) also recorded that 'human serum pseudocholinesterase' was less readily inhibited by fluoride with an acetylthiocholine substrate than with butyrylthiocholine.

Inhibition by fluoride is reversible both by dilution

and by dialysis against 0.9% sodium chloride (Heilbronn, 1965) and phosphate buffer (Cimasoni, 1966). Garry (1971) explained the differences in inhibition in tris and phosphate buffers by postulating that in the former the inhibitor was the fluoride ion and in the latter the fluorophosphate ion. However Cimasoni (1966) obtained comparable inhibitions of thiocholine ester hydrolysis in phosphate and veronal buffers. He concluded that the inhibition

"does not take place through the formation of complexes of fluoride with magnesium, phosphate or calcium; nor does the substrate play a role in the mechanism of inhibition. It can therefore be postulated that the enzyme molecules themselves are directly affected by the halide ions."

The statement that the substrate does not play a role is difficult to understand since the only experiments undertaken on this point did show different inhibitions with different substrates. Heilbronn (1965) considered many possible mechanisms including the hypothesis that trans-acylation occurred to fluoride rather than the hydroxyl ion and that the acyl fluoride was then the inhibitor. This could account for the more potent inhibitions found with the longer (butyryl) and bulkier (benzoyl) esters. She also considered inhibition of acylation due to a reaction of the inhibitor with the enzyme-substrate complex but finally recorded that an "interaction with a group in the active site, necessary for the acylation of the enzyme, seems indicated."

None of these postulated mechanisms however would

appear to account for the inversion of the phenotypic inhibitions. This occurs with all three substrates examined in this study at roughly the same fluoride concentration between 5×10^{-4} and 10^{-3} and is due to inhibition of the atypical variant reaching a plateau about this concentration. A possible explanation could be the presence of two inhibitory species, one a readily-formed fluoride complex and the other free fluoride ions, both equally inhibitory to the usual enzyme but only one a potent inhibitor of the atypical variant.

Speculations regarding these inhibitions far less their mechanisms must be tempered by the recent report (Brownson and Watts, 1973) that 5,5'-dithiobis-(2-nitrobenzoic acid) has an activating effect on cholinesterase. This activation is abolished at saturating concentrations of acetylthiocholine although it is still found with butyrylthiocholine under such conditions. Indeed it is even possible that this activation may be phenotype-dependent but this was not recorded.

This complication and that concerning the variation in absorption of the indicator ion with temperature were not taken into account in the temperature studies with the thiocholine esters. Only the non-enzymic hydrolysis of the substrate was allowed for in these experiments by including a control for each substrate at each temperature. The uncompensated complexities of this assay make calculation of temperature-activity constants a meaningless exercise. Even expressed as ratios referred to unity at 25°C as in Figures 62 to 64 probably yields erroneously high values

at the higher temperatures. However even without the necessary compensatory adjustments the temperature-activity relationships found for all three thiocholine ester substrates is sufficient to confirm that the phenotypic thermal responses found with a benzoylcholine substrate result from characteristic molecular alterations in the allelic cholinesterase isoenzymes.

Apart from this general confirmatory evidence any meaningfulness to be attached to these experiments can only be considered approximations, and that under the specific conditions employed. This does not invalidate these results from being used for screening purposes however (King and Ritchie, 1974). It would be quite valid by assays at 25°C and 45°C to infer that ratios of activity less than 1.5 with 5 mM acetylthiocholine (Figure 62), less than 1.55 with 5 mM propionylthiocholine (Figure 63) and less than 1.7 with 3 mM butyrylthiocholine (Figure 64) indicate a suxamethonium-sensitive phenotype. With the modified LKB 8600 Reaction Rate Analyser used in these studies, 100 cholinesterase assays can be performed in one hour. A realistic figure might be 600 assays per day, say 300 at 25°C in the morning then repeated at 45°C in the afternoon. This number of specimens could be processed readily using the LKB 2071 or the Hook and Tucker K40 sample-diluters and processing the teletype tape through a modest computer to obtain the ratios. The recently introduced Vitatron AKES which has a variable temperature control, a sampling-dispensing system and produces a print-out of results can increase the number of specimens screened to 400 per day

(McQueen and King, 1974).

The action of inhibitors may be used also to screen for suxamethonium-sensitive phenotypes. With 10^{-4} M dibucaine an inhibition of less than 40%, 38% or 50% with acetylthiocholine (Figure 47), propionylthiocholine (Figure 48) or butyrylthiocholine (Figure 49) respectively would indicate a sensitive phenotype. Less reliably, succinylcholine at 10^{-2} M sensitive phenotypes would give inhibitions less than 60%, 34% or 45% with acetyl- (Figure 50), propionyl- (Figure 51) or butyrylthiocholine (Figure 52) respectively. Chloride and fluoride do not provide sufficient differentiation to be of use even for screening.

Yet again the ratio of hydrolysis rates with two substrates may be employed although here the differentiation is less precise. However with 3 mM butyrylthiocholine and 5 mM acetylthiocholine an activity ratio of less than 1.65 (Figure 61) would suggest the need for further investigation of the specimen.

The experiments into the effect of the non-depolarising relaxants on the hydrolysis of acetylthiocholine by usual and atypical homozygous sera confirm the inhibitory action of pancuronium and tubocurarine noted earlier with a benzoylcholine substrate (page 92). There is however a relative difference in that an initial plasma level of 2.5 mM pancuronium is too low to have any effect on either variant (Figure 59) while an initial plasma level of 5 μ M tubocurarine would slightly inhibit the usual enzyme (Figure 60).

Particularly interesting is the inhibition of erythrocyte

acetylcholinesterase which with both pancuronium and tubocurarine closely resembles the behaviour of the atypical cholinesterase. Although both non-depolarising relaxants have an anti-acetylcholinesterase action this does not occur at concentrations achieved in the blood.

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4. Population Surveys

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During the course of the biochemical investigations reported herein, two extensive population studies were made, the first on a family or geographical basis, the second in a selected clinical setting.

Scalpay Survey

A patient admitted to the Lewis Hospital, Stornoway, suffered a "suxamethonium apnoea" and assay of his serum indicated cholinesterase activity of 260 mU/ml at 25°C, dibucaine number 19, fluoride number 22, chloride number 57 and scoline number 17. He was accordingly classified as phenotype $E_1^a E_1^a$ although in view of the low activity the presence of a silent gene was possible.

Family studies were suggested but this was difficult since the patient resided on a small, fairly remote island, Scalpay, off the Island of Lewis. His father was the "Elder" of the island which was the home of most members of his family. However, Dr. J.H. Burton, Surgical House Officer at the Lewis Hospital and Dr. J.W. Robertson, the local General Medical Practitioner took blood samples from 109 members of the family. As a result one brother and two sisters of the propositus were also classified as atypical homozygotes, two brothers as heterozygotes and one brother a usual homozygote. One further brother was at sea and no specimen was obtained and one sister was dead. The mother was also dead but the father was identified as a usual/atypical heterozygote.

Two neices of the propositus, daughters of a heterozygote brother, were classified phenotype $E_1^a E_1^f$ although in the absence of a blood sample from their mother, their certain

phenotype must remain in some doubt (p. 86 and Figures 24 and 26). A second-cousin was found to be phenotype $E_1^u E_1^u$ with a serum cholinesterase of 280 mU/ml which in a healthy individual again raises the possibility of the presence of a silent gene although there was no evidence for this in the immediate family and indeed the usual homozygote brother had serum cholinesterase activity of 1000 mU/ml. The phenotype relationship of the immediate family is shown in Figure 66.

Outside the immediate family of the propositus the relationship data received was somewhat less than satisfactory but a possible maternal aunt, a usual homozygote had serum cholinesterase activity of 2100 mU/ml, and twenty of the sera assayed had activities greater than the normal range of 600 to 1350 mU/ml. This might suggest segregation within the family of the C_5 variant but no attempt was made to confirm this. Although deriving from a second cholinesterase locus it has been shown that the C_5 variant apparently adopts the same inhibition characteristics as the variants at the first locus (Harris, 1970).

Despite the possible presence of these other variants in the population studied, in which incidentally there was no evidence of consanguineous matings, it had been hoped that in such an extensive family study the scatter of inhibitor numbers would be less than in the general population. Such a finding would lend support to the thesis (p. 86 et seq.) that the atypical gene, as currently accepted, is not a single entity but embraces several mutations. However the figures obtained and summarised in Table 16 were disappointing

Table 16

Summary of Results from Scalpay Survey

Phenotype	Number	Cholinesterase	Dibucaine Number	Fluoride Number	Chloride Number	Scoline Number
		(280 - 2100)				
$E_1^u E_1^u$	73	520 - 1740	79 - 83	56 - 66	9 - 19	88 - 93
$E_1^u E_1^a$	28	340 - 1320	54 - 69	45 - 52	21 - 30	63 - 70
$E_1^a E_1^a$	4	260 - 395	15 - 22	19 - 24	48 - 57	14 - 17

The values in parenthesis for cholinesterase activity of the usual homozygotes represent the true limits found. When these two values are deleted the narrower unbracketted range is obtained.

in this respect. The inhibitor numbers for the atypical homozygotes certainly show less scatter than the general population but this was from only four individuals.

Pregnancy Survey

The first patient suffered a "Scoline apnoea" at term in Glasgow Royal Maternity Hospital and a blood sample was sent for analysis. The results of cholinesterase 130 mU/ml, dibucaine number 29, fluoroide number 31 and chloride number 40 did not fit with any phenotype (Figures 2 and 29). The sample was haemolysed and although it was known that this did not invalidate the results a repeat specimen was requested. Analysis of this second specimen gave results for serum cholinesterase 105 mU/ml, dibucaine number 33, fluoride number 31 and chloride number 42. These second values were still anomalous but a provisional phenotype of $E_1^a E_1^a$ was given. A third blood sample was received five weeks later when the results of serum cholinesterase 435 mU/ml, DN = 44, FN = 35 and Cl N = 30 were obtained and indicated that the patient's phenotype was $E_1^a E_1^f$. Later assays confirmed this phenotype just as repeated assays of the first two specimens yielded the original anomalous results. The change in inhibitor numbers was paralleled by alterations in the temperature-activity relationships (Figure 67).

The second case originated in identical circumstances and the first determinations gave serum cholinesterase 175 mU/ml, DN = 30, FN = 34 and Cl N = 39 figures which did not correlate with any phenotype but most closely resembled those of $E_1^a E_1^a$. As before a later specimen

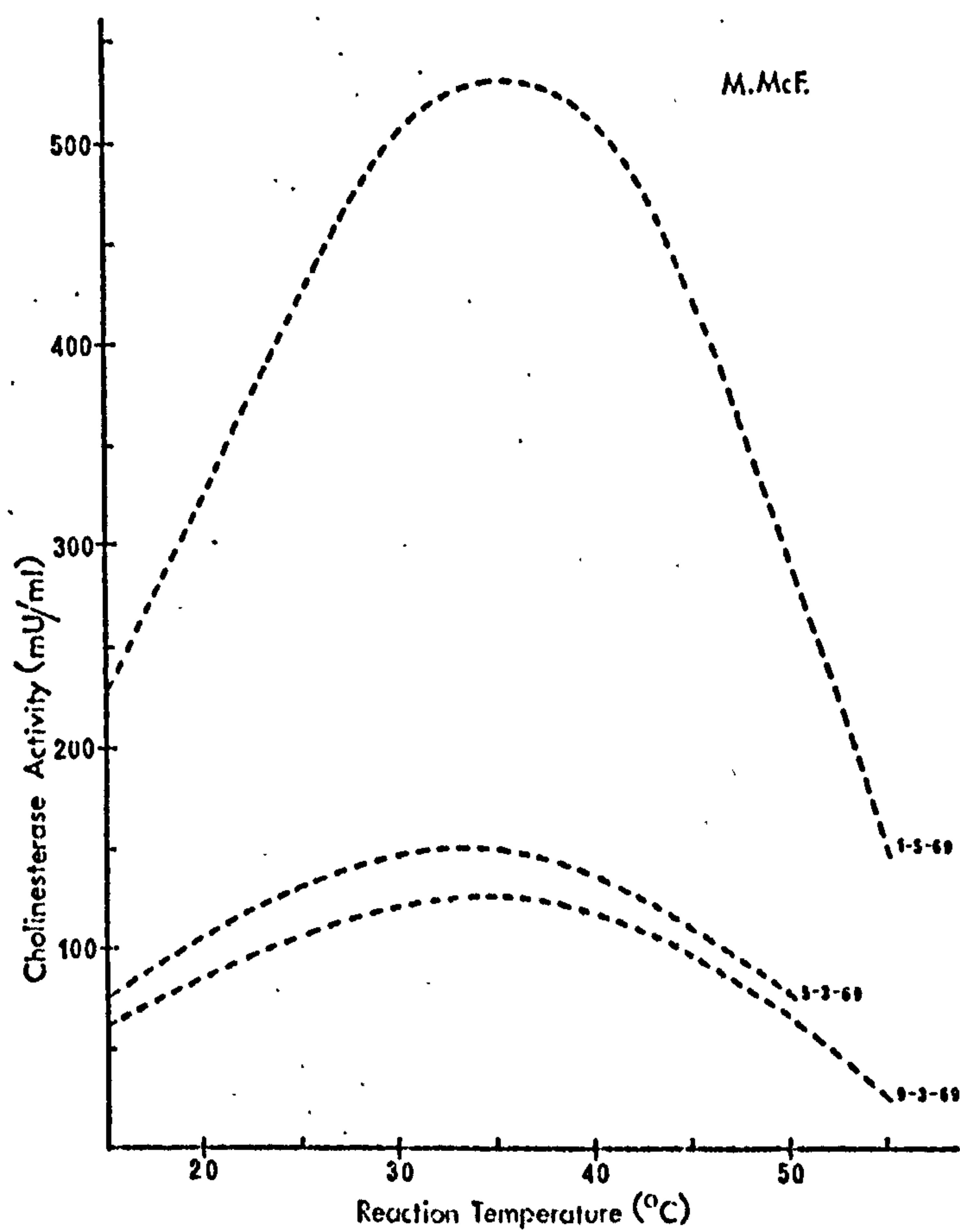


Figure 67. Temperature/activity curves for the serum cholinesterase of the first pregnancy case (page 123) at different times. The specimen dated 5.3.69 was taken on the day of delivery.

indicated a phenotype $E_1^a E_1^f$ with cholinesterase activity 375 mU/ml, DN = 45, FN = 37, Cl N = 31 and Scoline number 50.

Chloroform had been part of the anaesthetic regime in the first case and without any real conviction had been postulated as the cause of the anomalous inhibitor numbers. Chloroform was not employed with the second patient and the anomalous inhibitions were attributed generally to term pregnancy.

It was this background which gave rise to the comment (King, McQueen and Morgan, 1971) when the third case (patient M of Case 4, page 49) was presented that "assay of her serum at this time yielded the usual anomalous results associated with late pregnancy". These results were serum cholinesterase 365 mU/ml, DN = 54, FN = 32 and Cl N = 27 which corresponded most closely to phenotype $E_1^a E_1^f$. However later, post-partum figures for cholinesterase activity of 480 mU/ml and dibucaine, fluoride and chloride numbers of 64, 36 and 28 respectively indicated that she was in fact a fluoride-resistant homozygote, $E_1^f E_1^f$.

It was recalled (King, 1965) that the activity of oxytocinase, an aminopeptidase, increased in the blood plasma in pregnancy, reaching very high levels in the last trimester. It is the presence of this enzyme, which has overlapping substrate specificities with leucine aminopeptidase, which results in high activities of the latter enzyme being measured in blood serum in pregnancy (Green, Tsou, Bressler and Seligman, 1955; Bressler and Forsyth, 1959; Miller and Worsley, 1960).

It seemed possible that the aminopeptidase action

might be modifying the molecular structure of cholinesterase resulting in the anomalous inhibitions. Dr. L. Murray of the Glasgow Royal Maternity Hospital was therefore approached with the project of obtaining blood samples at various times during pregnancy for assay of leucine aminopeptidase activity, cholinesterase activity and inhibitor number to determine whether and at what point these last altered during pregnancy. Four hundred and fifteen blood samples from 148 patients were obtained over a period of fourteen months. Many of the specimens were single samples and from other patients only two blood specimens were received. However from 26 patients, five or more blood samples were obtained during pregnancy and in 13 cases post-partum specimens were also obtained. While analysis of the sera confirmed the pronounced increase in aminopeptidase activity already mentioned and the more modest decrease in cholinesterase activity (Friedman, Lapan and Taylor, 1961; Schnider, 1965; Robertson, 1966) in pregnancy, no change in inhibitor numbers was found. This was in agreement with the report by Hazel and Monier (1971) published about this time.

During this survey however a fourth term pregnancy apnoea with anomalous results was referred from Stobhill Hospital. The serum cholinesterase was 395 mU/ml, DN = 65, FN = 50, Cl N = 24 and SN = 78 which suggested a phenotype of $E_1^u E_1^a$ or less likely $E_1^f E_1^f$. Arrangements were made to phenotype close relatives and on this occasion the patient's sera gave values for cholinesterase of 570 mU/ml and of 76, 54, 21 and 86 for dibucaine, fluoride, chloride and scoline numbers respectively which indicated a usual/flouride-

resistant heterozygote $E_1^u E_1^f$. This phenotype agreed with those of parents and siblings.

It then became clear that the anomalies occurred in individuals possessing the fluoride-resistant gene. This accounted for the negative findings in the pregnancy survey in which only the usual and atypical variants had been encountered.

Attempts were later made with sera containing the fluoride-resistant cholinesterase to simulate the proposed mechanism in vitro by adding crystalline leucine aminopeptidase (Boehringer Corporation, leucine arylamidase). It was found however that addition of the enzyme caused an immediate alteration in all inhibitor numbers which did not further change significantly on prolonged incubation. The crystalline enzyme is supplied as a suspension in 3.2 M ammonium sulphate and it was found that addition of a simple 3.2 M ammonium sulphate solution resulted in the observed change in inhibitions.

Dialysis or ultrafiltration of the crystalline enzyme and subsequent solution in say 50% glycerol may remove this last effect but obviously a purification of cholinesterase as achieved by Das and Liddell (1970) would also seem indicated. Then, if changes in inhibitor numbers were noted on incubation with aminopeptidase or other proteolytic enzyme, an amino-acid analysis of the reaction mixture might give some insight into the alteration in the molecular structure of cholinesterase which brought about this change in kinetic behaviour.

5. Discussion, Conclusions and Prospects.

Discussion, Conclusions and Prospects

The studies undertaken in this project have produced results with applications and meaningfulness in the diverse but connected spheres of technology, biochemistry, physiology, pharmacology and genetics.

Technical and Clinical

For precise identification of sensitive cholinesterase phenotypes the assay method of Kalow and Lindsay (1955) employing a 50 μ M benzoylcholine substrate with inhibition by 10 μ M dibucaine (Kalow and Genest, 1957) and 50 μ M sodium fluoride (Harris and Whittaker, 1961) at 25°C has been shown to be adequate and indeed apparently essential. As King and Griffin (1974) have pointed out retention of this technique is certainly not on the grounds of ease or speed. The rate of hydrolysis is monitored at 240 nm which requires a fairly sophisticated ultraviolet recording spectrophotometer with temperature control, while the procedure is somewhat time-consuming and, by routine clinical biochemistry standards, demands more than the usual technical ability. However the studies on the variation of benzoylcholinesterase activity and the studies with other substrates have not revealed an alternative procedure capable of yielding precise categorisation of the phenotype. Nevertheless these investigations have disclosed means of screening for individuals at risk to the muscle-relaxant, suxamethonium.

By the use of thiocholine esters and the LKB 8600 Reaction Rate Analyzer it has been shown to be possible to screen 300 patients per day using the relative hydrolysis

rates of two substrates, dibucaine inhibition, or the ratio of hydrolysis rates at two temperatures. The first two of these techniques do not require the ability to alter the reaction temperature and can be achieved by the standard instrumentation, automated to a degree and performed quickly, simply and cheaply. These are the requirements for population screening.

The modifications carried out on the LKB instrumentation serve to underline the concept that the limits of endeavour in laboratory medicine must never be circumscribed by manufacturers. Only the laboratory worker knows what is required of him and only he knows what he must have to carry this out. The fact that the manufacturers of this instrument, following publicity attached to our modifications, now offer a variable temperature device as an optional extra, emphasises this point of view. As it is, recently introduced alternative analysers such as the Vitatron AKES system, the Union-Carbide Centrifichem and Aminco Rotachem, which have variable temperature capabilities, can be employed for cholinesterase screening using procedures based on any of the three principles outlined above.

However it is too easy to be blinded by dedication to a single goal. The ability to carry out population screening for cholinesterase variants would appear to be possible as a result of these studies. The question to be considered now and which should have been posed at the outset, is whether screening for suxamethonium sensitivity is desirable and worthwhile. Obviously it is desirable to have information concerning the sensitivity of a patient to a drug. At

present biochemical investigations are only undertaken after the apnoeic episode has occurred and in a few laboratories such as these, close relatives are subsequently offered phenotyping. This is a very limited screening. As it is, the ability to screen 300 patients per day would cover all surgical patients in the Glasgow Royal Infirmary group of hospitals. Indeed it can be estimated that if screening were to be limited to patients receiving suxamethonium, 300 per day would provide such discriminatory cover to the whole West of Scotland Region, embracing a population of some three million. Since about one in three thousand of the population is of a predictably sensitive phenotype (Lehmann and Liddell, 1969) then on the average the proposed screening programme would reveal one such patient every ten days of operation. During this same period however one other patient would suffer a suxamethonium apnoea which in our present state of biochemical knowledge and anaesthetic practice could not have been predicted (Lehmann and Liddell, 1969). At its very best then a fifty per cent success rate is all that can be expected from a screening programme for suxamethonium sensitive individuals. Were the apnoeic episodes to carry a similar percentage of fatal outcomes, indeed any significant percentage of fatalities, then the answer to whether screening is worthwhile would never have to be given for the question would not be asked. However today the phenomenon is well understood by all practising anaesthetists and while it is the cause of some inconvenience and even prolonged, intensive attention it does not constitute a serious major problem either to patient or

anaesthetist. Under these circumstances it is debatable whether mass screening is more desirable and worthwhile than the limited selective screening currently undertaken in these laboratories.

It has been amply shown above that in the clinical setting under discussion, that of suxamethonium apnoea, the standard procedure for identifying the serum cholinesterase variants has little to recommend it to the analyst. That it does precisely define the cholinesterase phenotype is the sole reason for its retention because the results obtained are not always readily correlated by the anaesthetist with the degree of sensitivity. Generally it can be accepted that the lower the cholinesterase activity and the lower the dibucaine and fluoride numbers the longer will be the period of paralysis. However, even excluding the 50% of all apnoeas which occur in the usual homozygote or heterozygotes and hence are not predictable, as was seen from Figure 14 the serum activity of many suxamethonium-sensitive individuals is in the normal range of this standard procedure at 25°C. The studies herein recorded have indicated that a false impression is obtained of the activities of the most sensitive phenotypes by assay at 25°C and a true index is only obtained at 37°C as shown in Figure 15.

The second lack of correlation between enzymic parameters and apnoeic response occurs with the fluoride-resistant homozygote, $E_1^f E_1^f$. This sensitive phenotype is categorised by a dibucaine number, and also chloride and scoline numbers, of the same order as the non-sensitive

usual/atypical heterozygote, $E_1^u E_1^a$. Again the explanation lies with the assay temperature used. Figure 35 showed that the response to temperature of the dibucaine inhibition of the $E_1^f E_1^f$ phenotype is more pronounced than the others. At 37°C, the temperature which the patient exhibits the sensitivity, the dibucaine number is similar to that of the predictably sensitive $E_1^a E_1^f$ phenotype.

These temperature studies on the inhibition of benzoylcholine hydrolysis have clearly shown that accurate temperature control is essential for precise phenotype categorisation, which only adds to the technical strictures of the method. On a more general topic the finding of the different temperature-activity relationships of the allelic cholinesterase isoenzymes provides important factual evidence on the vexed problem of a standard temperature for clinical enzyme assays (King, 1972). In this controversy one of the first questions to be considered is whether the actual assay temperature and the standard temperature need be one and the same. If there is no compulsion for this then it is possible to conduct an assay at the temperature giving greatest advantages for each individual enzyme and then convert to the standard temperature. This reduces to the question of whether temperature correction factors are feasible and valid. In the present instance, although lengthy and cumbersome, following determination of the phenotype, the appropriate correction factors can be used. This however could not be done for varying proportions of isoenzymes as encountered with lactate dehydrogenase or alkaline phosphatases. The studies with the thiocholine

esters, also show that the correction factors vary with the substrate employed. From these facts it was concluded that at best temperature conversion factors can only give approximations and their present indiscriminate use should be avoided. As a necessary consequence of this, the assay and standard temperatures must be the same.

The principle has been applied further and on the temperature-activity differences between human serum enzymes and the enzymes in commercial quality control sera, limits of valid use of the latter have been described (King, Henderson and Morgan, 1972).

The failure to precisely classify the allelic variants at any temperature other than 25°C raises questions apart from those already discussed under the appropriate sections, and in particular the basic question of defining the variants.

Genetics

As the work of Lehmann and co-workers on haemoglobin (Lehmann and Huntsman, 1966; Lehmann and Carrell, 1969) and Harris and colleagues on enzymes (Harris, 1970) has shown, protein polymorphism is the rule rather than the exception. A non-lethal mutation occurs, is passed on and a balance is eventually achieved with the remainder of the population. Such mutations occurring initially in the DNA coding may result in a simple substitution of a single amino-acid at any point in the polypeptide chain with variations down to an incomplete or nonsense chain. The latter possibilities of course are examples of "silent genes". Depending upon the change in properties attending upon the simple substitution and its point in the chain diverse alterations

in the three-dimensional protein conformation will result with consequent alterations in stability and enzymic characteristics. In our present state of knowledge of enzymes, unlike haemoglobin, we are not capable generally of differentiating such mutations by specifying the actual change in molecular structure. Instead such variants are differentiated by relatively crude procedures such as electrophoresis or by precipitation, inactivation or inhibition by reagents, antibodies or physical means such as heat. The means by which the mutations are differentiated is of necessity therefore the terms in which the mutations are defined. In the present instance the first allelic cholinesterase isoenzyme to be differentiated from the usual was termed the "atypical" and was defined by its relative resistance to inhibition by dibucaine under specific assay conditions. Sodium fluoride not only achieved the same but revealed a further sub-differentiation (Harris and Whittaker, 1961) attributable to another allelic variant and for obvious reasons termed the fluoride-resistant enzyme. These mutations were therefore defined in terms of the inhibition of 50 μ M benzoylcholine hydrolysis in phosphate buffer of pH 7.4 at, or near, 25°C by 10 μ M dibucaine or 50 μ M fluoride. In view of the interdependence of all factors influencing enzyme activity it would be most unlikely therefore if the definition of the allelic isoenzymes were retained when the conditions of determination were altered.

Since dibucaine and succinylcholine act as simple competitive inhibitors it is possible by manipulating substrate and inhibitor concentrations to maintain the

definitive inhibitions at other temperatures and with other substrates. However these studies have shown (Figures 56 - 58) that the inhibition by fluoride is complex and this in conjunction with the profound effect of temperature on the inhibition (Figure 39) is responsible for the failure to precisely differentiate the mutations other than under the definition conditions.

By the use of other inhibitors such as chloride (Whittaker, 1968b; King and Dixon, 1970), formaldehyde (Whittaker, 1969) and n-butyl alcohol (Whittaker, 1968a) the presence of further variants has been postulated. From these present investigations the correlations between succinylcholine inhibition and others (Figures 23 to 28) tend to support this and reasoning from other similar protein mutations strongly advises that a large number of structurally similar cholinesterases exist. This proposition did not gain any support from the family survey on the island of Scalpay. However if the original postulate is correct then starting with the mother and father it is possible that two different usual and two different atypical genes were segregating between the propositus and his siblings. Only in the homozygote, usual or atypical, can the genetic compositions be identical but the numbers, four atypical homozygotes is too small to draw any conclusions from the narrow scatter of inhibitor numbers obtained (Table 16).

The postulate that there is a quantitative genetic control of serum cholinesterase (Wetstone, Honeyman and McComb, 1965) in addition to the well established qualitative control receives no support from these investigations.

Certainly the mechanisms which control type need not control amount but if the mechanism controlling quantitative production is uncoupled from that controlling type then it is to be expected that the rate of synthesis of the two allelic proteins will be separately affected. The thermodynamic data herein derived suggests that quantitatively there is equal gene synthesis. This can only be accepted as a mean or average assessment however since the values used in the calculations were such themselves. Reasoned extrapolation from other enzymes would make it seem more likely that the quantitative differences observed in serum cholinesterase activities in a normal population are mediated by environmental and hormonal rather than genetic influences. As discussed earlier the numerically large values attached to serum cholinesterase activity are a reflection of its "serum specificity" and the relative range span, that is, the ratio of upper to lower limits of normal activity, is small compared to other enzymes.

Pharmacology

The problems associated with the mixing of depolarising and non-depolarising relaxants are well summarised by Wyllie and Churchill-Davidson (1972). However the literature does not appear to contain any report of the inhibition of cholinesterase by the non-depolarising relaxant, pancuronium, and thus finding would theoretically appear to further complicate anaesthetic practice.

This initial finding of cholinesterase inhibition by pancuronium related to benzoylcholine hydrolysis but was also found with an acetylthiocholine substrate and would

therefore appear to be a true anticholinesterase action. While an antiacetylcholinesterase activity has also been shown (Figure 59) this effect only occurred in vitro at concentrations not likely to be obtained in blood. However it is stated that a concentration of 10^{-5} M pancuronium is required at the neuromuscular junction for blockade (Goodman and Gilman, 1970) and at this concentration there is some inhibition of acetylcholinesterase with consequent depolarising action. The manufacturer's data (Organon Laboratories) states that the maximum effect is evident after 2-2½ minutes and, depending upon dosage and anaesthetic techniques, lasts about 45 minutes. On the results of the experiments described herein it would certainly seem ill-advised to administer pancuronium before suxamethonium since the pancuronium inhibition, particularly of the usual homozygote, would reduce the hydrolysis of the suxamethonium. Even administration of pancuronium after suxamethonium but before the effects of the latter had passed off, would seem to be less than ideal.

On these in vitro experiments it is difficult to understand why a partial "dual block" is not obtained by administration of pancuronium alone. When both relaxants are administered it is apparently standard anaesthetic practice to give suxamethonium first and one is assured that no untoward reactions are obtained under these circumstances. In only two cases of "Scoline apnoea" among the many which are of non-predictably sensitive phenotype, one a usual homozygote and one an atypical heterozygote was it found that pancuronium had been administered and in the accepted sequence.

Biochemistry and Physiology

Paigen (1971) stated "The most sensitive and accurate test for changes in protein structure is relative thermostability. The probability that a random aminoacid substitution produces a protein with altered thermostability is more than 50%. This probability is higher than for any other test, and the necessary measurements can generally be made with considerable accuracy in dilute solutions of crude enzyme".

By "thermostability" Paigen obviously referred to denaturation by incubation at a set temperature and the sensitivity of this has been demonstrated by Walsh, Lowell and Neurath (1966) in two carboxypeptidase A allelomorphs differing only in the antepenultimate C-terminal aminoacid residue and by Langridge (1968) in induced mutations of the β -galactosidase of E.Coli. However as far as the writer is aware the experiments described herein are the first in which differential temperature analysis has been employed in the recognition of enzyme molecular forms. The principle has considerable potential. King (1972) has suggested that it could be applied to other enzyme species and in fact has used it in differentiating human macroamylase from the normal α -amylase (Henderson, King and Imrie, 1973; Imrie, King and Henderson, 1973).

The studies on the hydrolysis rates of the thiocholine esters would indicate that the relative affinity for butyrylthiocholine shown by the usual enzyme is not shared by the fluoride-resistant variant and less so by the atypical isoenzyme. Indeed the last shows almost equal activity with acetylthiocholine and butyrylthiocholine (Figure 61) almost

a tendency towards the substrate specificities of acetylcholinesterase. The similarity in the inhibition by pancuronium of the atypical homozygote and erythrocyte acetylcholinesterase (Figure 59) offers support to this premise. If therefore as has been suggested (Jamieson, 1963; Koelle, 1963) the physiological role of cholinesterase is not dissimilar to that of acetylcholinesterase then the atypical homozygote is at no disadvantage in normal circumstances. Indeed the enzyme will function at substrate concentrations not only inhibitory to acetylcholinesterase but even to the usual variant although it is highly improbable that such concentrations will be found in vivo.

The principle of differential substrate analysis illustrated by the above thiocholine ester studies (Figure 61) has been employed with pyruvate and 2-oxobutyrate as substrates for lactate dehydrogenase (Wilkinson, 1972). In these laboratories this has been extended by the use of hydroxypyruvate (McQueen and King, 1971a,b) and glyoxylate (King, Young and Naghizadeh, 1973; Young, Naghizadeh, King and Morgan, 1973).

Speculations and Prospects

In their erudite review of the mechanism of hydrolysis by cholinesterase Davies and Green (1958) critically examined the kinetic evidence available and convincingly concluded that histidine was present in the anionic active site of cholinesterase.

With regard to the allelic cholinesterase isoenzymes the difference in substrate and inhibitor specificities is most evident with positively charged compounds (Kalow and

Davies, 1958; Davies, Marton and Kalow, 1960) indicating differences in the anionic sites. Harris and Whittaker (1963) have pointed out that the differential inhibition by fluoride and high concentrations of chloride does not necessarily conflict with this view.

Most mutations result from single-point DNA transitions or transversions and Epstein (1967) has pointed out that this generally results in an aminoacid substitution which conserves the properties of the polypeptide. In this particular instance this proposition gains support from the lack of difference in electrophoretic mobilities of the variants which in itself could suggest the substitution of an aminoacid with unionised side chain being substituted by another or by one the side chain of which is uncharged at pH 8.6. The imidazole side chain of histidine is uncharged at pH 8.6 the pH at which protein electrophoresis is usually performed. Equally well the substitution could be for histidine and the electrophoretic separation of the variants at pH 9.7 (Liddell, Lehmann, Davies and Shank, 1962) would require the substituent side chain to have a less negative charge than imidazole at this pH.

Photo-oxidation in the presence of methylene blue which specifically decomposes histidine and tryptophan was used in demonstrating a functional role for imidazole groups in chymotrypsin, (Cohen and Oosterbaan, 1963). This procedure or the blocking of the imidazole ring by selective sulphonylation below pH 5 should elucidate this possibility. If histidine has a function in the cholinesterase active site then photo-oxidation or sulphonylation would destroy

or impair the activity of usual homozygote sera. If substitution of the histidine is the mutation which results in the inhibitor-resistant variants then such homozygous sera would be unaffected, unless of course using the photo-oxidation process, tryptophan were the substituent.

Accepting the above circumstantial evidence that histidine is a functional component of the active site of cholinesterase and further that the allelic variants arise from its substitution there are seven possible one-point transitions and transversions which could occur in its genetic coding (CA - C/U). These mutations could result in substitutions by aspartic acid (GA - C/U), tyrosine (UA - C/U), arginine (CG - C/U), proline (CC - C/U), leucine (CU - C/U), asparagine (AA - C/U) and glutamine (CA - G/A). Tryptophan is not a possible substitution from a single-point mutation. Of the possible substituents it can be reasoned that the first four would not have uncharged side-chains at pH 8.6 and less negative charges than imidazole at pH 9.7. The aliphatic side chain of leucine and the amides of asparagine and glutamine do fulfill both conditions.

The finding of anomalous inhibitor numbers at term pregnancy in patients possessing the fluoride-resistant gene is important as this constitutes the first recording of a cholinesterase mutation outside the control of the structural gene. Since this modification apparently only occurs in the fluoride-resistant enzyme this is further proof that there is a structural difference between this variant and the others. Further if the postulate is correct that the fluoride-resistant molecule is modified by the action of

oxytocinase then since the aminopeptidase only splits off N-terminal residues it is in this position that the fluoride-resistant variant differs from the usual and atypical iso-enzymes.

Again, oxytocinase has a high affinity for N-terminal cystine; leucine aminopeptidase for leucine but also for phenylalanine, tyrosine, histidine and tryptophan. If therefore the future studies proposed with leucine aminopeptidase (page 126) do not yield similar alterations in inhibition characteristics as found in term pregnancy this could constitute evidence that the N-terminal residue was not an amino-acid readily attacked by leucine aminopeptidase.

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